



Characterization of the underlying mechanisms of adjuvanticity of cyclic di-nucleotides

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr. rer. nat.)
genehmigte
D i s s e r t a t i o n

von Rimma Libanova
aus Tschernowzy / Ukraine

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eingereicht am: 23.11.2011
mündliche Prüfung (Disputation) am: 16.02.2012

Druckjahr 2012

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikationen

Libanova,R.; Ebensen,T; Schulze,K; Yevsa,T; Morr,M; Guzmán CA. (2011). "Bis-(3',5')-cyclic dimeric adenosine monophosphate: Strong Th1/Th2/Th17 promoting mucosal adjuvant." Vaccine 29(32): 5210-20.

Libanova,R.; Ebensen,T., Schulze,K.; Bruhn,D.; Norder,M.; Yevsa,T.; Morr,M.; Guzmán,CA. (2010). "The member of the cyclic di-nucleotide family bis-(3', 5')-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant." Vaccine 28(10): 2249-58.

“What we know is a drop, what we don’t know is an ocean.”

Isaac Newton

Acknowledgements

The work presented in this thesis was performed in the Vaccine Research Group at the Helmholtz Centre for Infection Research in Braunschweig, Germany. Many people have contributed to this work either directly or indirectly, co-workers, colleagues and friends.

I am extremely grateful to my advisor, Professor Dr. Carlos A. Guzmán, for giving me the chance to work in his lab. Furthermore, I thank him for his guidance, inspiration and continuous support, throughout my graduate research in the field of Vaccinology. I thank him for the conversations and new ideas that have helped to improve my skills in becoming a better scientist.

I sincerely thank Prof. Dr. Gursharan S. Chhatwal from the Department of Medical Microbiology at the HZI and the Technical University of Braunschweig for acting as the first referee.

Furthermore, I would like to thank the members of my thesis committee, Professor Dr. Jochen Hühn from the Department of Experimental Immunology, and Professor Dr. Lothar Jänsch from the Department of Cellular Proteome Research for their advice, time and helpful suggestions.

I would also like to thank Dr. Michael Morr for the synthesis of the cyclic di-nucleotides, as well as Dr. Hans-G. Genieser and Dr. Frank Schwede from the BIOLOG Life Science Institute Bremen for providing us the 2'-Fluo-AHC-c-di-AMP compound.

Furthermore, I would like to thank the my VAC group members Dr. Thomas Ebensen, Dr. Kai Schulze, Dr. Pablo Becker, Dr. Jenny Debary, Dr. Beata Zygmunt, Dr. Peggy Riese, Angelika Höhne, Ulrike Bröder, Elena Reinhard, Alex Cabrera, Dr. Miriam Nörder, Sebastian Weissmann, Kirsten Scholz and Stephanie Trittel for the scientific and laboratory support they offered me during all the years, and being my friends with over the years at HZI, for making my graduate study a very enriching experience.

Finally, I want to thank my parents, Sebastian Hennies as well as Ilse-Maria and Gerd Hennies for their endless love, support and patience. Thank you for always believing in me and the encouragements when needed.

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Abbreviations

ABTS	2,2'- azino - bis (3 - ethylbenzthiazoline – 6 sulfonic acid) diammonium salt
AEC	3 - amino - 9 - ethylcarbazole
APC	allophycocyanin
APCs	antigen presenting cells
BAL	bronchial alveolar lavage (lung lavage)
β-Gal	beta-galactosidase
BSA	bovine serum albumin
cAMP	cyclic adenosine-monophosphate
CBA	Cytometric Bead Array
CD	cluster of differentiation
cDC	conventional DC
c-di-AMP	cycle di-adenosine-monophosphate
c-di-GMP	cycle di-guanosine-monophosphate
c-di-IMP	cycle di-inosine-monophosphate
CFSE	Carboxyfluorescein diacetate succinimidyl ester
Ci	Curie
cLN	cervical lymph nodes
ConA	concanavalin A
Cpm	counts per minute
(BM-)DCs	(bone marrow derived-) dendritic cells
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
e.g.	<i>exempli gratia</i>
ELISA	Enzyme – Linked Immunosorbent Assay
ELISPOT	Enzyme – Linked Immunospot Assay
ERK	extracellular-signal-regulated kinases
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein - isothiocyanate
Flt3L	Fms-like tyrosine kinase 3 ligand
GM-CSF	granulocyte-macrophage colony stimulating factor
h.i.	heat inactivated
HRP	horseradish peroxidase

i.e.	<i>id est</i>
IDCs	lymphoid DCs
IFN	interferons
IFNAR	interferon- α/β receptor
Ig	immunoglobuline
IL	interleukine
i.n.	intranasal
KO	knock out
LAMP2	lysosomal-associated membrane protein 2
LN	lymph node
M	molar
M Φ	macrophages
MACS	magnetic-activated cell sorting
MALP-2	macrophage activating lipopeptide of 2 kDa
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
mDCs	myeloid DCs
MDFI	median fluorescence intensity
MHC	major histocompatibility complex
Minute	min
mM	mini molar
NALT	nasal-associated lymphoid tissue
NK	natural killer
OD	optical density
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pDC	plasmacytoid DC
PE	phycoerythrin
PEG	polyethylene glycol
PFA	paraformaldehyde
PRR	pattern-recognition receptor
PMSF	phenylmethanesulfonylfluoride
RT	room temperature
SEM	standard error of the mean
TCR	T cell receptor

TLR	Toll-like receptor
Th	T helper

ABSTRACT

The development of vaccines remains a valuable tool for the prevention of many infectious diseases. First vaccines were either attenuated or inactivated organisms. Subunit vaccines were then introduced as more refined formulations, exhibiting improved safety profiles. However, purified antigens tend to be poorly immunogenic and often require the use of adjuvants to achieve adequate stimulation of the immune system. Some of the latest players arrived to the field of adjuvant development are the cyclic di-nucleotides. These are ubiquitous prokaryotic intracellular signalling molecules that are not produced by eukaryotic cells, which make them to act as danger signals. Previous studies demonstrated that bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) exhibits potent adjuvant activity when administered by systemic or mucosal route. The first part of this thesis is focused on the evaluation of two other members of this family, namely bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP) and bis-(3',5')-cyclic dimeric inosine monophosphate (c-di-IMP), as mucosal adjuvants. It is demonstrate that the novel cyclic di-nucleotides also exhibit strong activity as mucosal adjuvants. Intranasal immunization of mice with c-di-GMP, c-di-AMP or c-di-IMP co-administered with the model antigens β -galactosidase (β -Gal) or ovalbumin (OVA) resulted in significantly higher serum antigen-specific IgG titres and a higher production of antigen-specific secretory IgA (sIgA) in different mucosal territories, as compared with vaccination with antigen alone. In addition, strong cellular immune responses were also observed against both the β -Gal protein and a peptide encompassing its MHC class I-restricted epitope, respectively. The ratio of β -Gal specific antibodies IgG1 versus IgG2a and the profiles of the cytokines secreted by *in vitro* re-stimulated splenocytes suggested that a balanced Th1/Th2/Th17 response pattern is promoted by all three cyclic di-nucleotides. However, c-di-AMP showed more potency in terms of its *in vivo* and *in vitro* activities, being able to activate murine and, particularly, human dendritic cells (DCs) and macrophages. Therefore, the second part of this thesis was focused on the identification of the targeted cell types and putative molecular mechanism of action of c-di-AMP. The obtain results showed a strong activation of different DC subsets and a specific production of IL-12 and IL-10 by conventional DCs (cDCs) after c-di-AMP activation. Functional studies performed with c-di-AMP showed the adjuvant accumulating intravesicular in early endosomes and lysosomes of DCs as well as the activation of the MAPK pathways p38 α and ERK in DCs and macrophages (M Φ s). Moreover, *in vivo* studies on cell specific IFN- β reporter mice revealed that the adjuvant is also able to trigger a type I IFN response in DCs and monocytes/ M Φ s.

1. CHAPTER: INTRODUCTION

1.1 The immune system

1.1.1 *Innate immune system*

One of the most important functions of the immune system is the identification and clearance of a wide variety of dangerous entities, such as microbial pathogens, thereby protecting the host against diseases [1]. This is implemented through a network of cells, tissues, and organs, which are working together to defend the body against attacks by viruses, bacteria or parasites. To this end, there are two major tightly integrated systems: the innate immune response (ancient non-specific self-defence system) and the adaptive immune system (agent-specific response). The stimulation of an efficient immune response against any given infectious agent requires a harmonic interaction between components of these two systems [2].

1.1.1.1 *Pathogen recognition in the innate immune response*

The innate immune system is the first line of the defence system against microbial pathogens such as bacteria, fungi and viruses. This system includes physical barriers, such as the skin and epithelia, which are equipped with additional chemical features that help to restrain microbial invasion, e.g. antibacterial peptides, fatty acids, enzymes and low pH [3]. However, once the pathogen has crossed this first line of defence, different innate cells, such as dendritic cells (DCs), macrophages (MΦ), granulocytes (e.g., eosinophils, neutrophils), and natural killer (NK) cells can recognize them. Since microbial pathogens are evolutionarily different from their hosts, they display conserved pathogen associated molecular patterns (PAMPs), which in turn can be specifically recognized by pattern recognition receptors (PRRs). These receptors represent a large group of conserved receptor molecules like intracellular and extracellular TLRs (Toll-like receptors), and the cytosolic NLRs (NOD-like receptors) and RLRs (retinoic acid inducible gene-1 (RIG-I)-like receptors) expressed in different types of immune cells, such as DCs [4-6]. After binding of pathogenic components, PRRs trigger the release of pro-inflammatory cytokines (e.g. interferons), thereby leading to the stimulation of an adaptive immune response [7, 8]. By using these effector mechanisms, the innate immune system is able to rapidly and non-specifically react stopping invading pathogens, however, it cannot induce pathogen-specific and long lasting protective immunity. Nevertheless, the activated innate immune system represents the critical bridge for the

subsequent stimulation of specific adaptive immune responses against those microbes that pose a threat to the host [9]. The establishment of an adaptive response requires time, but it is tailored to specific agents and results in long-term memory. Thus, the innate responses prevent immediate death upon infection, whereas the more sophisticated subsequent adaptive response cannot only clear the pathogens, but also prevent infection and/or disease following re-infection.

1.1.1.2 *The role of dendritic cells in the immune system*

DCs are the main professional antigen-presenting cells (APCs), which derive from bone marrow (BM) progenitor cells. They play a critical role in translating the message from the innate immune system, being responsible for the initiation of adaptive T and B cell responses (Figure 1). Using PRRs for microbial and inflammatory products, DCs respond to internalized antigen or PAMPs, either by phagocytosis or endocytosis. After binding of pathogenic components to PPRs, immature DCs undergo a maturation process characterized by the up-regulation of antigen presenting molecules including major histocompatibility complex (MHC) class I and II, adhesion molecules such as CD11c, CD54 (ICAM-1), and co-stimulatory molecules including CD80 (B7.1), and CD86 (B7.2) [10]. CD86 is up-regulated earlier during DC maturation, as compared to the CD80 marker [11]. An additional marker of matured DCs in humans is CD83 [12]. Following activation, DCs can induce innate and adaptive immune responses, supporting the fight against pathogens. The two best-studied defence systems in DCs are the production of protective cytokines like interleukin-10 (IL-10), IL-12, IL-18 and type I interferons (IFNs), and the activation and expansion of innate and adaptive lymphocytes [8, 9]. Targeting the translation of innate into adaptive information by DCs offers new perspectives for manipulating the immune system for clinical benefit [8].

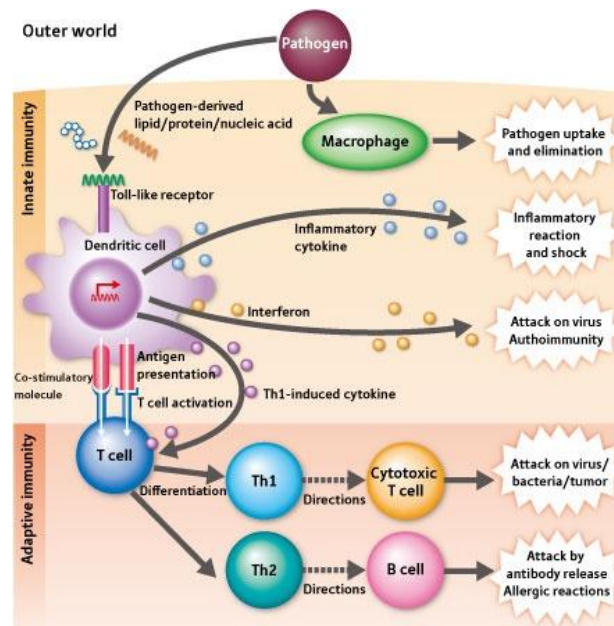


Figure 1: DCs bridging innate and adaptive immunity.

Adopted from Tsuneyasu Kaisho, RIKEN Research, 2007 [13]

There are two main types of DCs in the murine system: the conventional or classical DCs (cDCs) and the plasmacytoid DCs (pDCs). The cDCs consist of different subtypes, including CD8⁺ cDCs (myeloid DCs, mDCs) and CD8⁻ cDCs (lymphoid DCs, IDCs), which have a dendritic shape and exhibit typical DC functions such as antigen uptake, processing, and presentation [14]. They express different toll like receptors (TLRs) such as TLR-2, -3, -4, -7 and -9. After encountering different natural ligands or pathogens for these TLRs, cDCs become activated and mature into APCs, secreting cytokines (e.g. IL-12) and activating T helper cells, as well as B cells, by presenting them antigens derived from the pathogen. By flow cytometry, murine cDC are characterized as CD11c⁺ CD11b⁺ B220⁻ [15]. Importantly, pDCs express a different profile of the microbial pattern recognition TLRs than cDCs. Human and mouse cDCs can express TLR-1, -2, -3, -4, -5, -7, and -8 [16, 17] while pDCs uniquely express TLR-7 and TLR-9 in the endosomes [18-20]. Since, it is unconventional in the mouse system to isolate DC subpopulations or precursors directly from peripheral blood or tissue, due to the small sample size, DCs are mostly generated from bone marrow precursors with granulocyte-macrophage colony-stimulating factor (GM-CSF) or the FMS-like tyrosine kinase 3 receptor ligand (Flt3L). GM-CSF has led to models of the development of cDCs and the stimulation with Flt3L leads to a more appreciated model for studying the origin of a mixed pDC and cDC population [14, 21].

Despite several distinct subtypes of DCs, they all have the ability to engulf antigens in their environment by endocytosis using phagocytosis, receptor-mediated endocytosis, and pinocytosis [22-24]. Phagocytosis is an essential component of the innate immune response.

Phagocytic vacuoles acquire degradative and antimicrobial properties through a complex series of interactions with endomembrane. This process, collectively termed phagosome maturation, culminates in the fusion of phagosomes with lysosomes, yielding a hybrid organelle enriched in hydrolytic enzymes, reactive oxygen species and antimicrobial peptides that promote the killing and degradation of internalized microorganisms [25].

1.1.1.3 *Type I interferons and their effect on immune cells*

The type I IFNs consist on a number of structurally homologous proteins including 13 IFN- α subtypes, a single IFN- β , and other less well characterized proteins. However, the role of type I IFNs in the immune system has been characterized in detail only for IFN- α and IFN- β [26]. Type I IFNs can be produced by almost any cell type in the body in response to microbes by a number of transmembrane and cytosolic receptors. However, in response to viral infections, type I IFN is mainly produced by pDCs [27, 28]. Monocytes, which are able to differentiate into DCs, could also produce type I IFN, although much less than pDCs, after interaction with infectious agents [29]. All type I IFN species interact with the same IFN- α/β cellular receptor (IFNAR), the activation of which triggers several signalling cascades. The first signalling pathway described after activation by type I IFNs was the JAK-STAT (Janus activated kinase - Signal Transducer and Activator of Transcription) pathway [30]. But there are evidences that several other signalling elements and cascades are required for responses to type I IFNs, such as the MAPK pathway and others [31]. The different effector functions of type I IFNs result in an improved innate immune response, which in turn leads to an enhanced adaptive immune response. Furthermore, IFN- α/β up-regulate the expression of the IL-12 receptor β 2 chain on CD4⁺ T cells, thereby facilitating their differentiation into IFN γ producing Th1 cells in response to IL-12.

1.1.2 ***Adaptive immune system***

In contrast to the innate immune system, the adaptive immune system has evolved to recognize microorganisms that are able to escape the non-specific defence systems of the host. This is achieved by the stimulation of a highly specific and sophisticated response. The adaptive immune system manifests exquisite specificity for its target antigens, due to antigen-specific receptors expressed on the surfaces of T and B lymphocytes. The antigen-specific receptors of the adaptive response are assembled by means of somatic rearrangement of germline gene elements to form intact T cell receptor (TCR) and immunoglobulin (Ig) genes [2]. In addition to the specificity, the most important difference is

the development of memory, which allows a very rapid response of antigen specific effector cells upon a second encounter with the relevant antigen from a pathogenic organism.

T lymphocytes also arise from BM, but unlike the B cells, T cells migrate to the thymus gland for maturation. The T cell expresses a unique antigen-binding molecule, the so-called TCR, which recognizes only antigens bound to MHC molecules. MHC molecules are polymorphic (genetically diverse) glycoproteins found on cell membranes. There are two major types of MHC molecules: class I MHC molecules, which are expressed by nearly all nucleated cells of vertebrate species, and class II MHC molecules, which are expressed only by professional antigen-presenting cells. MHC class I molecules present antigen fragments to cytotoxic T cells and will bind to CD8 on cytotoxic T cells, and MHC class II present antigen fragments to Th cells by binding to the CD4 receptor on the Th cells. The adaptive immune response can be divided into two main arms, namely, cellular and humoral immunity [32].

1.1.2.1 *Humoral immunity*

Humoral immunity is the major defence mechanism against foreign antigens, such as extracellular pathogens and toxins. The humoral immune response is mediated by the secretion of antibodies, which are produced by B plasma cells, which are derived from B lymphocytes. B cell expresses a unique antigen-binding receptor on its membrane after leaving the BM where they mature. When a naïve B cell encounters an antigen that matches its membrane-bound antibody for the first time, the binding induces antigen specific B cells to rapidly divide into many clones of plasma cells and memory B cells. Plasma cells produce antibodies in a form that can be secreted and has little or no membrane-bound capacity [32, 33].

Antibodies or Ig, are not only receptors on the surface of B cells, but in addition are soluble proteins in extracellular fluids (sera, plasma, lymph, mucus, etc.), which are able to bind to antigens. This antigen-antibody binding contribute to host protection by three main ways: neutralization, opsonisation and complement activation. Neutralization is important in preventing bacterial toxins from entering the cells. The coating of the surface of a pathogen to enhance the phagocytosis is called opsonisation. Complement activation results in complement protein binding to the pathogen surface, and subsequently opsonisation of the pathogen by binding complement receptors on phagocytes or direct lysis [34].

In general, B cells need two signals to become active and to secrete the specific antibodies. The first signal is the cross-linking of the B cell-surface bound Ig by antigen. However, most antigens are T cell dependent, i.e. T cell help is required for maximal antibody production, and the second activation signal is delivered by a Th2 cell. The T cell

dependent antigens are taken up by surface-bound antibodies, processed and finally antigenic peptides are presented on B cells via MHC class II to antigen-specific Th2 cells. These T cells then provide co-stimulation signals, which trigger B cell proliferation and their differentiation into plasma cells and memory cells. The first class of antibodies secreted after B cell activation are belonging to the IgM isotype. Subsequently, class switching to IgG, IgA, IgD and IgE, and the generation of memory B cells occur in response to cytokines [35]. IgG and IgA are the predominant and highly specific antibody classes. Most B cells secreting these Ig classes have been selected for increased affinity of Ag-binding in germinal centers, where they underwent affinity maturation by somatic hypermutation. IgG is the most abundant Ig class in plasma in part due to its longer life halftime, whereas IgA is the most abundant Ig in secretions [32].

1.1.2.2 *Cellular immunity*

Cell-mediated immunity (CMI) involves the activation of antigen-specific T lymphocytes and their effector functions. The principle role of CMI is to detect and eliminate cells that harbour intracellular pathogens. CMI can also recognize and eliminate cells, such as tumour cells, which have undergone genetic modification. Both antigen-specific and non-specific cells can contribute to CMI. Antigen-specific cells include CD8⁺ cytotoxic T lymphocytes (T_C cell or CTLs) and cytokine-secreting CD4⁺ T cells or Th cells. Non-specific cells include NK cells and non-lymphoid cell types such as macrophages, neutrophils and eosinophils [32]. Importantly, there are other specialized T cells that express CD4 and CD25 known as regulatory T cells (Treg cells), or suppressor T cells, which are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus [36].

CTLs represent essential effector cells, which recognize and are able to kill cells expressing foreign peptide antigens in association with MHC class I molecules. The response of CTLs is antigen specific and contact dependent. For this purpose, CTLs contain membrane-bound cytoplasmic granules filled with proteins, including perforins and granzymes, which are capable to cause cell lysis. Furthermore, CTLs are able to protect the body by destroying body cells displaying epitopes of foreign antigens on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumour antigens. In addition, activated CTLs cells secrete a variety of cytokines that influence the function of other bystander cells involved in innate and adaptive immune responses, such as IFN- γ [37, 38].

CD4⁺ Th cells are another important cell population of the adaptive immune system and can be differentiated into different types of effector subsets with different immunological functions. The earlier discovered Th1 and Th2 CD4⁺ T cells proliferate in response to antigen stimulation, thereby leading to the production of IL-2 and its receptor through an autocrine loop, which in turn stimulates antibody production and macrophage activation. IFN- γ -producing Th1 cells were presumed to take part in the phagocytic process and the intracellular defence against viruses and bacteria. In contrast, Th2 cells, which secrete IL-4, IL-5 and IL-13, orchestrate non-phagocytic and extracellular defence against helminths and take part in allergic reactions. Th2 cells initiate the humoral immune response by activating naïve antigen-specific B cells to produce antibodies. They are essential in determining B cell antibody class switching, in the activation and growth of T cells and in maximizing bactericidal activity in phagocytes, such as macrophages. These responses are important in the defence against parasites and mucosal pathogens. The discovery of the third type of Th cells, the Th17 lineage, came later from studies performed using murine models of experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA). Formerly, the autoimmune reactions observed in these models were wrongly attributed to the stimulation of a Th1 immune response. This resulted from the observation that EAE or CIA was ablated by treatment with neutralizing antibodies specific for the p40 subunit of IL-12, the main cytokine responsible for Th1 cells development. However, subsequent studies demonstrated that IL-23 shares with IL-12 the p40 subunit [39]. Thus, the IL-12 heterodimer is composed of IL-12p40 and IL-12p35 subunits, whereas the IL-23 is formed by IL-12p40 and IL-23p19. Later studies using knockout (ko) mice showed that the disease develops in IL-12-ko mice, but not in IL-23-ko mice [40]. These pioneer studies resulted in a blooming interest in the field for elucidating the underlying mechanisms of the development and function of Th17 cells. Currently it is known that IL-23 is not important for development of Th17 subsets, but it is also required for the maintenance of this CD4⁺ T cell sub-population [41]. The development of Th17 cells is also under negative regulation of IL-2. This diversity in Th function and their role in influencing other immune cells pointed to the importance of these cells during the course of natural infections. For example, in the Human immunodeficiency virus (HIV) infection the virus infects CD4⁺ cells. This in turn leads to a massive decrease of functional CD4⁺ T cells, thereby resulting in AIDS symptoms [35, 42-44].

1.1.3 *Mucosal immune system*

The mucosal tissues represent the first line of defence against infection, since it constitutes the main entry site for most microbial pathogens. Therefore, components of the highly specialized innate and adaptive immune system protect the mucosal surfaces and body interior from potential injuries. This local immune system contributes to almost 80% of all immune cells. The cells are accumulated in, or transit between the mucosa-associated lymphoid tissues (MALT), which as a whole represents the largest mammalian lymphoid organ (Figure 2) [26]. MALT includes the intestinal mucosa (Gut-Associated Lymphoid Tissue, GALT) and the respiratory tract (Nasal-Associated Lymphoid Tissue, NALT; and Bronchus-Associated Lymphoid Tissue, BALT). NALT is composed of a bell-shaped structure located in the nasal passages above the hard palate of rodents and other mammals [45, 46]. In mice NALT organogenesis begins soon after birth and is dependent on several factors, including various cytokines and chemokines, as well as environmental cues [47]. In humans, NALT-like structures are evident at a very young age, but disappeared by the age of two years, whereas the Waldeyer's ring, which also includes nasopharyngeal lymphoid tissues, persists throughout life [48]. The architecture of NALT is structured like lymph nodes, organized into discrete compartments of immature B and T-lymphocytes and antigen-presenting DCs [49, 50].

The mucosal immune response has three main functions: (i) protection of the mucosal surface against colonization and invasion by dangerous microbes, (ii) prevention of the uptake of non-degraded antigens, including foreign proteins derived from ingested food, airborne matter and commensal micro-organisms, and (iii) interference with the development of potentially harmful immune responses to these antigens if they reach the body interior [51, 52]. The MALT functions independently from the systemic apparatus. It is formed by anatomically defined lymphoid micro-compartments (Peyer's patches, lymph nodes, appendix, tonsils and adenoids), which serve as mucosal inductive sites where adaptive immune responses are initiated [53].

An important characteristic of the mucosal adaptive immune response is the local production and secretion of IgA and IgM dimers and oligomers in both mice and humans. These IgA and IgM polymers originate from the interaction of IgA or IgM monomers with the J chain (IgA+J and IgM+J), a polypeptide synthesized by antibody-secreting cells. In addition, the J chain interacts with the polymeric Ig receptor (pIgR). The polymeric Ig receptor is expressed basolaterally as membrane secretory component (mSC) on secretory epithelial cells [54]. The secretory component of sIgA and sIgM protects the Ig from being degraded by proteolytic enzymes. The resistance to host proteases makes sIgA uniquely suited for functioning in mucosal secretions to protect the body against adhesion and entry of

pathogens at mucosal sites. The induction of sIgA is dependent on Th2 cells that produce IL-4 and IL-10, thereby prompting B cell Ig class switch to IgA and differentiation into IgA producing plasma cells (Figure 2) [52]. The number of active subclasses for IgA is different among different species. The mouse has only one, but in humans there are two subclasses, IgA1 and IgA2, which are characterized by a differential distribution in the body. Indeed, the circulating IgA pool is comprised mostly of IgA1, whereas the mucosal IgA pool contains both IgA1 and IgA2 [55]. IgA2 is particularly abundant at sites colonized by microbes, including the distal intestinal tract and the urogenital tract [56, 57].

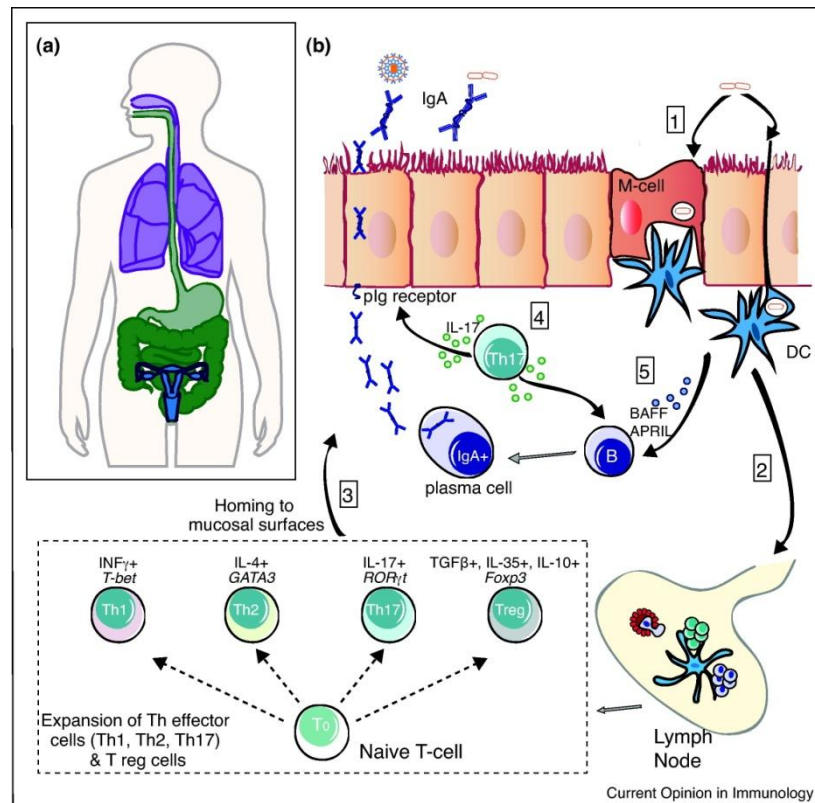


Figure 2: Mucosal Immunity.

Mucosal surfaces (A) constitute the largest interface between the body and the external environment, including the respiratory (purple), gastrointestinal (green) and genital (blue) tracts. **Mucosal immune response (B)** pathogen surveillance is mediated by specialized antigen transport cells (M-cells) and antigen processing cells (DCs) (1). Mucosal DCs are particularly important at initiating adaptive immune responses by migrating to the draining lymph node and mediating the expansion of antigen-specific naïve T-cells into Th subsets (Th1, Th2, Th17, or T regulatory cells). (2), involving an up-regulation of transcription factors and lineage-defining cytokines (IFN-γ, IL-4, IL-17, TGFβ, IL-35, and IL-10). Expanded T cell subsets will home back to mucosal surfaces to perform their effector functions (3). Th17 cells and IL-17 expression can up-regulate polymeric Ig (pIg) receptor humoral defense mechanism mucosal CTL expression and IgA class switching, enhancing IgA secretion (4). In addition, soluble factors secreted by DCs and epithelial cells can promote T-cell Independent IgA class switching (5). Increased IgA production and translocation through epithelial cells hinders pathogen invasion and promotes immunity at mucosal surfaces. Adopted from Lawson et al., *Current Opinion in Immunology*, 2011 [58]

The CTLs present in the mucosal tissue also have a crucial role in the clearance or containment of mucosal viral infections [59]. Protective mucosal immune responses are most effectively induced by mucosal immunization via nasal, oral, rectal or vaginal routes [60]. The nasal mucosa is an important inductive site of the mucosal immune system, since this site is the first port of entry for inhaled pathogens.

The nasal mucosa is an important compartment within the mucosal immune system, since this site is the first port of entry for inhaled pathogens. Furthermore, there is an induction of CTLs in distinct mucosal tissues. More interesting, there is a significant degree of compartmentalization linking specific mucosal inductive sites with particular effective sites. Depending on the choice of vaccination route an effective immune responses can be induced at the desire sites. Thus, rectal immunization evokes strong local IgA antibody responses in the rectum, whereas nasal or tonsilar immunization in humans results in antibody responses in the upper airway mucosa and regional secretions (salvia, nasal secretions). This is attractive for the development of vaccines against sexual transmitted diseases [52, 61].

1.2 Vaccination

Vaccination works by inducing a pathogen-specific immune response through the administration of vaccine antigens, which enable the stimulation of memory cells that could later recognize and respond to a similar organism before it could cause disease. Vaccination has been and still is one of the most important public health interventions in history. The practice of immunizing to confer protection against disease can be traced to as early as the 7th century when Indian Buddhists drank snake venom to induce immunity, possibly through a toxoid-like effect. Immunization (i.e. the act of protecting against disease by introducing an antigen into the body to induce immunity) against smallpox probably began early in the 1022 to 1063 AD in central Asia and then spread to China and west Turkey [62]. Smallpox used to be a common disease throughout the world and 20 to 30% of infected persons died from the disease. Smallpox was responsible for 8 to 20% of all deaths in several European countries in the 18th century. Other experiments followed, but Edward Jenner's could show in the 18th century, that immunization with the less dangerous cowpox could protect against infection with smallpox. This experiment inspired indeed the word *vaccination*, which is derived from *vacca*, the Latin word for cow [63, 64]. In the 19th century Louis Pasteur developed the concepts of attenuation, modification through passage, renewed virulence and the need to replace person-to-person vaccination with something safer, more consistent, and less likely to transmit other diseases [65].

Since those pioneering efforts, vaccines have been developed for many diseases that were once major afflictions of mankind. The incidence of diseases such as diphtheria, measles, mumps, pertussis (whooping cough), rubella (German measles), poliomyelitis, and tetanus has declined dramatically as vaccination has become more common. Despite the fact that vaccination is the most cost-effective weapon to prevent infectious diseases [66], there are still many diseases for which vaccines are not available. Every year, millions of individuals die throughout the world from malaria, tuberculosis, AIDS, viral hepatitis, respiratory or gastrointestinal infections, just to name a few, because there are no effective vaccines or the available vaccines cannot reach those in need. For example, it is estimated by the World Health Organization (WHO) that every day 15,000 people, mainly young adults become infected with HIV (e.g. Figure 1). An effective vaccine could have an immense impact in the control of the spread of HIV/AIDS [66].

1.2.1 *Vaccines*

Recent advances in immunology and molecular biology has led to the development of novel vaccines with satisfactory efficacy and safety profiles. The identification of T and B cell epitopes has also enabled immunologists to design new vaccine candidates, which are able to stimulate both arms of the immune system. Vaccines can be roughly classified into different types. Live attenuated vaccines, such as some paediatric vaccines (e.g. measles, mumps, rubella, oral polio and BCG) stimulate the immune system via a transient infection caused by replicating live organisms without causing clinical illness. However, they are able to cause diseases in immune compromised individuals. Thus, a safer vaccination approach is based on the use of inactivated organisms (e.g. inactivated vaccines against polio or whooping cough) or non-replicating subcellular components, the so-called subunits (e.g. subunit vaccines against polio and hepatitis B virus). Well defined vaccines based on selected subcellular components are very attractive, since the immune stimulus is maximally directed to the molecule that is relevant for stimulating protection. However, the identification of an essential immunogenic component which is able to promote protective responses is complicated and many pathogens have evolved antigenic variation as escape mechanism. In addition, these vaccines are usually less reactogenic than whole-cell vaccines, but also considerably less immunogenic. Therefore, they often require additional components known as adjuvants in the formulation, which stimulate the immune system and potentiate/modulate antigen-specific responses, while having little or no antigenicity on their own [67-71].

1.2.2 Adjuvants

Adjuvants (*adjuvare* (latin) = to help) have been traditionally used to increase the magnitude of an adoptive response to a vaccine, but a second role for adjuvants has become increasingly important: namely, guiding the type of adoptive response to produce the most effective form of response for each specific pathogen. Therefore, there are different mechanisms of action which are reported to contribute to the biological activity of adjuvants incorporated into vaccines [69]. They can (i) provide an antigen depot, thereby creating an antigenic reservoir for slow release; (ii) facilitate the antigen targeting to antigen presenting cells; (iii) promote antigen processing and presentation; and (iv) create a microenvironment conducive towards modulation and enhancement of the elicited immune response [72, 73]. Adjuvants also provide a danger signal that tells the immune system that a particular antigen cannot be ignored. The use of particular adjuvants offers the possibility to improve the efficiency of mucosal applied vaccines [74]. An ideal adjuvant should be stable, biodegradable, cheap, poorly immunogenic by itself and able to promote strong immune responses of a well-defined type [72, 75]. Adjuvants can be broadly divided into two groups based on their principal mode of action: delivery systems and immunostimulatory molecules. Delivery systems (mineral salts, oil-in-water emulsions, virosomes and liposomes) are mainly enhancing the uptake of antigens by APCs, whereas immunostimulatory molecules induce a rapid activation of innate immunity. Many of the immunostimulators are sensed by various members of the TLR family, a subclass of PPRs. They may also exert dual or overlapping effector functions [72, 76]. Nevertheless, this distinction is arbitrary, since a considerable body of experimental evidence is accumulating, demonstrating that even adjuvants which were traditionally considered as promoters of delivery (e.g. aluminium salts [alum]) exhibit important immune modulatory properties.

Overall, several hundred of natural and synthetic compounds have been identified, but only a few are licensed for human use, such as alum, oil-in water emulsions (MF59 and AS03), and monophosphoryl lipid A (MPL; a non-toxic derivative of the TLR-4 agonist lipopolysaccharide) plus alum (AS04) [68, 74]. Others are in widespread experimental use or in late stage clinical development (Table 1) [72].

It is clear that novel adjuvants will be required to enable the successful development of innovative vaccines. Both components, antigen and adjuvants, are of critical importance in order to induce an immune response that will protect against diseases for which traditional approaches have failed, such as HIV and Hepatitis C virus (HCV) [77, 78].

Table 1: Adjuvants licensed for human vaccines or under clinical development

Adjuvant	Formulation	Type of Immune Response	Vaccines	Reference
Alum	Non-crystalline gels based on aluminum oxyhydroxide or hydroxyphosphat	Th2	HBV*, HAV*, HPV*, diphtheria-tetanus, haemophilus influenza B, inactivated polio virus, etc.	[79, 80]
MF59, AS03	Squalene-based oil-in-water emulsion	Th1 and Th2	HSV, HIV, influenza (Fluad), pandemic flu (Pandemrix)	[80-82]
AS04	MPL absorbed on alum	Th1	HBV, HAV, HPV, Flu	[80]
CTB	Cholera toxin B subunit	Th1 and Th2	Orally delivered cholera vaccine (Dukoral, Shanchol, Orochol)	[83, 84]
Advanced Development				
Poly(I:C)	Synthetic derivatives of dsRNA	Th1, CD8 ⁺ T cells	Flu, cancer	[72, 85, 86]
MPL and formulations (AS01, AS02)	MPL and QS-21	Th1 and Th2	Malaria, TB*, cancer	[80, 87]
CpG*	Oligonucleotide + alum, oligonucleotide + MF59, oligonucleotide	Th1, CD8 ⁺ T cells (when conjugated)	HBV, malaria, HVC, cancer	[88, 89]
ISCOMS and ISCOMATRIX	Phospholipids, cholesterol, QS-21	Th1 and Th2, CD8 ⁺ T cells	HCV, HSV*, HBV, HIV* influenza, malaria, cancer	[80, 89]
IC31	KLK* with ODN	Th1, B-cell responses	Influenza, TB, HCV*	[90, 91]

*HBV = Hepatitis B virus; HAV = Hepatitis A virus; HPV = Human papillomavirus; HSV = Herpes simplex virus; TB = tuberculosis; HIV = human immunodeficiency virus; HCV = Hepatitis C virus; CpG = CpG oligodeoxynucleotides; QS = QS-21; KLK = anti-microbial peptide

1.2.3 Mucosal vaccination and adjuvants

Mucosal immune responses are most efficiently induced by administration of vaccines onto mucosal surfaces (e.g. intranasally, orally). This route of immunization does not only induce cellular and humoral immune responses at the site of vaccine application, but also throughout the body. In addition, mucosal immunizations offer several advantages over parenteral administration, e.g. easy administration, lower costs, possibility of self-administration, avoidance of needle-stick injuries, low rates of local side effects and high acceptance (i.e. better compliance) by the public [52, 92]. This makes them particularly amenable for implementation in mass vaccination campaigns.

Despite these advantages, only a few mucosal vaccine candidates were tested, and even less reached the market. One mayor problem is still the poor immunogenicity of the antigens when administrated by mucosal route due to mechanical removal and structural modification by extreme pH and/or enzymatic degradation [93, 94]. To overcome these problems many groups tried to develop adjuvants which are able to promote efficient antigen specific immune responses at both systemic and local levels. However, there are only a

handful of molecules exhibiting this property [95, 96]. In addition, there are serious concerns about the safety profile of some of these compounds [97]. Thus, there is still a strong need for new candidate adjuvants.

1.2.4 The cyclic di-nucleotides as mucosal adjuvants

Cyclic di-nucleotides are second messenger molecules of bacterial and archaeal origin, which exert their activity by regulating several biological processes. Among them can be cited bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) and bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP) (Figure 3).

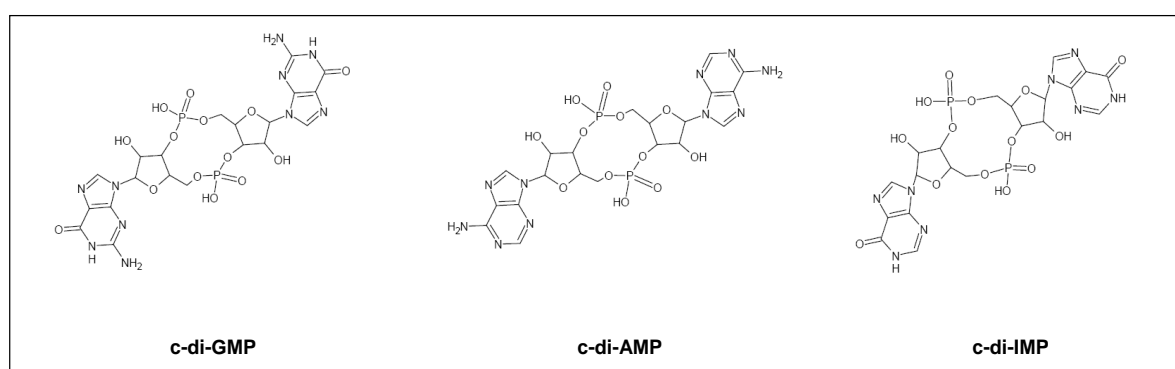


Figure 3: Chemical structures of cyclic dinucleotides.

The c-di-GMP was first identified as a cellulose synthase activator in *Glycanocetobacter xylinus* [98]. Furthermore, it is essential for the regulation of biofilm formation, motility, virulence gene expression and other critical aspects of physiology in a number of pathogens [99-108]. High levels of c-di-GMP enhance biofilm formation and repress virulence factor expression and motility, whereas low levels of c-di-GMP repress biofilm formation and induce virulence factor expression and motility [104, 109]. Karaolis *et al.* showed that exogenous c-di-GMP reduces *in vitro* cell-cell interactions and biofilm formation of *Staphylococcus aureus* [110] and also demonstrated that treatment with c-di-GMP attenuates *S. aureus* infection *in vivo*, reducing bacterial loads in a mouse infection model [111]. Recent reports have described another compound of the cyclic di-nucleotide family, c-di-AMP, which represents a novel second messenger, which signals for DNA integrity in *Bacillus subtilis* during sporulation [112]. During the time in which the experimental work of this thesis was carried out, c-di-AMP was also identified as molecule secreted by *Listeria monocytogenes*, which is able to trigger the cytosolic innate host response [113, 114]. However, it remains still unclear the evolutionary advantage resulting from c-di-AMP expression for *Listeria* spp. Nevertheless, the role played by c-di-AMP in *Listeria* spp. seems to be totally different from the function in *B. subtilis*, thereby indicating

diverse evolutionary pathways and roles of this second messenger in bacteria. Since they seem to be crucial for bacterial survival, it can be assumed that these molecules could serve as danger signals recognized by the host immune system. It can be argued that other structurally related compounds could act as danger signals able to evoke an immune response in the host. An example of this is the bis-(3',5')-cyclic dimeric inosine monophosphate (c-di-IMP) (Figure 3), which can be synthesized as a derivative of the parental compound c-di-AMP through adenosine-deamination.

1.3 Aims of the study

The first aim of this doctoral thesis was to perform a comparative evaluation of the immune modulatory properties of c-di-GMP, c-di-AMP and c-di-IMP to select the most promising candidate adjuvant for further development. The second aim was to perform in depth *in vitro* experiments using cells of murine and human origin, as well as *ex vivo* and *in vivo* studies in mice to identify the target immune cells and underlying mechanisms of action of the resulting compound.

2. CHAPTER: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemical and reagents

Table 2: Chemical components and reagents

Chemical agent or reagent	Manufacturer/Distributor
⁵¹ Chromium (chromate solution)	Amersham, Germany
ABTS (2,2'-azino-bis (3 ethylbenzthioazoline-6-sulfonic acid)	Sigma-Chemie, Germany
AEC substrate kit (3-amino-9-ethyl-cabazole)	BD Pharmingen™, USA
Acetic acid (CH ₃ COOH)	Merck, Germany
Ampuwa®	Serumwerk, Germany
Avidin-HRP (horseradish peroxidase) conjugated (ELISPOT)	BD Pharmingen™, USA
Ammonium chloride (NH ₄ Cl)	Merck, Germany
Beta-galactosidase protein (β-Gal)	Roche, Germany
BSA (Bovine serum albumin)	Sigma-Chemie, Germany
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Invitrogen, Germany
Citric acid –1- hydrate (C ₆ H ₈ O ₇ * H ₂ O)	Riedl-de-Haën, Germany
DMEM medium (low Glucose, 0.1 g/L)	Gibco, UK
DMEM medium (high Glucose, 4.5 g/L)	Gibco, UK
DMF (N,N-dimethylformamide)	Sigma-Aldrich, Germany
DMSO (dimethyl sulfoxide)	Sigma-Aldrich, Germany
Ethylenediaminetetraacetic acid (EDTA)	Fluka, Switzerland
Ethanol 100%	Fluka, Switzerland
FCS (fetal calf serum, South America)	Greiner Bio-One, USA

FLT3L (Fms-like tyrosine kinase 3 ligand)	BD Pharmingen™, USA
Formaldehyde ≥ 36.5%	Riedl-de-Haën, Germany
Gentamycine (50mg / ml in deionised water)	Gibco, UK
GM-CSF (granulocyte-macrophage colony stimulating factor)	BD Pharmingen™, USA
L-Glutamine	Gibco, UK
Isofluran®Curamed vet inhalation anesthetic	Essex Tierarznei, Germany
Isopropanol	Merck, Germany
LPS (Lipopolysaccharide)	Sigma-Aldrich Germany
M-CSF (macrophage colony stimulating factor)	BD Pharmingen™, USA
2-Mercaptoethanol (50 mM)	Gibco, UK
[methyl- ³ H] Thymidine solution	Amersham, Germany
OVA (Ovalbumin)	Sigma-Aldrich Germany
Paraformaldehyde (PFA)	Merck, Germany
Penicillin/Streptomycin (100 units/ml Penicillin G sodium; 50 µg/ml streptomycin sulphate in 85% Saline)	Gibco, UK
PMSF (phenylmethylsulfonylfluoride)	Carl Roth GmbH, Germany
Potassium chloride (KCl)	Fluka, Switzerland
Potassium hydrogen carbonate (KHCO ₃)	Merck, Germany
Potassium dihydrogen phosphate (KH ₂ PO ₄)	Carl-Roth, Germany
RPMI 1640 medium (L-glutamine)	Gibco, UK
Saponin	Sigma-Aldrich, Germany
Sodium acetate (CH ₃ COONa)	Merck, Germany
Sodium carbonate (Na ₂ CO ₃)	Carl-Roth, Germany
Sodium chloride (NaCl)	Carl-Roth, Germany

Sodium hydrogen carbonate (NaHCO_3)	Merck, Germany
Sodium hydroxide (NaOH)	Carl-Roth, Germany
Streptavidin – horseradish peroxidase conjugate (ELISPOT)	BD Pharmingen™, USA
Streptavidin –HRP (horseradish peroxidase) conjugated (ELISA)	Sigma-Aldrich, Germany
tri-Sodium citrate	Merck, Germany
Triton X100	Carl Roth GmbH, Germany
Trypsin-EDTA (1x) in HBSS	Gibco, UK
Tween 20	Carl-Roth, Germany

2.1.2 Solutions and buffers

Table 3: Solutions and buffers

Solution / buffer	Composition
ABTS-solution	0.3 g/l ABTS in 0.1 M citric acid
Acetate solution (0.1 mM)	148 ml acetic acid 0.2 mM to 352 ml of 0.2 mM sodium acetate; adjust volume to 1 l with water, pH 5.0
ACK lysis buffer	0.1 mM EDTA; 1 mM KHCO_3 ; 155 mM NH_4Cl ; pH 7.3
Carbonate buffer for ELISA	1.59 g/l Na_2CO_3 ; 2.93 g/l NaHCO_3 ; pH 9.6
FACS-buffer	1% BSA/PBS (w/v)
Lavage buffer	5% FCS/PBS (v/v)
Paraformaldehyde	2% (w/v) in PBS; pH 7.0 (tissue fixation)
PBS (phosphate-buffered saline)	2.7 mM KCl; 1.8 mM KH_2PO_4 ; 137 mM NaCl; 10 mM Na_2HPO_4 ; pH 7.4

2.1.3 Cell culture media

Table 4: Cell culture media

Medium	Supplements
D-MEM for DC	10% FCS heat inactivated; 100 Units/ml penicillin; 50 µg/ml streptomycin; 1mM L-glutamine; 50 µg/ml gentamycine
D-MEM for MΦ	10% FCS heat inactivated; 100 Units/ml penicillin; 50 µg/ml streptomycin; 1mM L-glutamine; 5% horse serum; 20% L929-conditional medium (M-CSF)
Freezing medium	FCS heat inactivated + 10% DMSO
RPMI complete	RPMI 1640 supplemented with 10% FCS heat inactivated; 100 units/ml penicillin; 50 µg/ml streptomycin; 1 mM L-glutamine; 2.5 x 10 ⁻² M 2-mercaptoethanol

2.1.4 Mice and cell lines

Table 5: Mice

Strain	Commentary
BALB/c (H-2 ^d) mice	Harlan-Winkelmann GmbH, Borcheln, Germany
C3H/HeJ LPS non-responder mice	Jackson Laboratory, USA
OT-I, OT-II and DO.11.10 mice	Bred in animal facility of the Helmholtz Centre for Infection Research (HZI)
Type I IFN reporter mice (IFN-β ^{+/-Δβ-luc} , LysM, CD11c ^{cre} , CD4 ^{cre} , CD19 ^{cre} , IFNAR ^{-/-})	Bred in animal facility of the HZI

Cell lines

The **J774A.1** (mouse monocytes-macrophages) cell line was obtained from the DSMZ (German National Resource Centre for Biological Material). The cell line was established from a tumour in a female BALB/c mouse in 1968; J774A.1 cells were described to synthesize lysozyme and interleukin-1 and to have receptors for Ig and complement. They are relatively small cells growing adherently; adherent cells will round up after a few days in

culture [115]. The cells were cultured in DMEM with 5% heat-inactivated FCS, penicillin/streptomycin, 2 mM glutamine and 2.5×10^{-2} M 2-mercaptoethanol. They were incubated at 37°C (5% CO₂) in a humidified atmosphere, having a doubling time of 17 hours.

2.1.5 Solution and buffer used for ELISA

Table 6: Solution and buffers for ELISA

Solutions / buffers	Compositions
Coating buffer	Carbonate buffer
Blocking buffer (ELISA)	PBS – BSA 3% (w/v)
Washing buffer (ELISA)	PBS – Tween 0.1% (v/v)
Dilution buffer	PBS-BSA 3% (w/v)
ABTS/H ₂ O ₂ solution	0.03% (v/v) H ₂ O ₂ in ABTS-solution

2.1.6 Solution and buffer used for ELISPOT

Table 7: Solutions and buffers for ELISPOT

Solutions / buffers	Compositions
Coating buffer	1 x PBS
Blocking solution	RPMI 1640 medium + 10% FCS + 1% penicillin-streptomycin-L-glutamine
Wash buffer I	1 x PBS + 0.05% Tween-20
Wash buffer II	1 x PBS
Dilution buffer	1 x PBS + 10% FCS
Substrate solution	333.3 µl of AEC stock solution (100 mg AEC in 10 ml DMF) +10 ml 0.1 mM acetate solution + 5 µl H ₂ O ₂

2.1.7 Antibodies

Table 8: Murine surface antibodies used for FACS analysis

Antibodies/Conjugate	Clone	Species	Dilution	Company
Anti-mouse CD16/CD32 purified (FcR-block)	2.4G2	mouse	1:500	BD Pharmingen™, USA
Anti-mouse CD3 APC/PE	145-2C11	hamster	1:500/1:200	eBioscience, USA

Anti-mouse CD4 APC / PE-Cy7	RM4-5	rat	1:500/1:1,500	eBioscience, USA
Anti-mouse CD8 APC	53-6.7	rat	1:500	eBioscience, USA
Anti-mouse CD11b PE / eFluor®450	M1/70	rat	1:300 / 1:800	BD Pharmingen™, USA
Anti-mouse CD11c PE-Cy7	N418	hamster	1:800	BD Pharmingen™, USA
Anti-mouse CD25 PE	PC61.5	rat	1:300	eBioscience, USA
Anti-mouse CD40 FITC	3/23	rat	1:100	BD Pharmingen™, USA
Anti-mouse CD45R (B220) PE-Cy5	RA3-6B2	rat	1:300	eBioscience, USA
Anti-mouse CD54 FITC (ICAM-1)	3E2	hamster	1:100	BD Pharmingen™, USA
Anti-mouse CD62L FITC (L-Selectin)	MEL-14	rat	1:1,000	eBioscience, USA
Anti-mouse CD80 APC (B7-1)	16-10A1	hamster	1:1,200	BD Pharmingen™, USA
Anti-mouse CD86 PE (B7-2)	GL1	rat	1:500	BD Pharmingen™, USA
Anti-mouse H-2K ^d FITC (MHC-I)	SF1-1.1	mouse	1:100	eBioscience, USA
Anti-mouse I-A/I-E eFluor450 (MHC-II)	M5/114.15.2	rat	1:200	eBioscience, USA
Live/Dead Far Red kit	-----	-----	1:1,000	Invitrogen
Anti-mouse CD205 (DEC205) PerCP-eFluor 710	205yekta	rat	1:200	eBioscience, USA

Table 9: Murine antibodies used for FACS analysis against intracellular targets

Antibodies/Conjugate	Clone	Dilution	Company
IL-10 Alexa700	JES5-16E3	1:100	BD Pharmingen™, USA
IL-12/23p40 Alexa647	C17.8	1:400	eBioscience, USA

Table 10: Human antibodies used for FACS analysis

Monoclonal Antibodies [all obtained from mouse]	Clones	Dilution	Company
Anti-human CD11c V450	B-Ly6	0.5 µl/5 x 10 ⁵ cells	BD Pharmingen™, USA
Anti-human CD14 PE	M5E2	5 µl/5 x 10 ⁵ cells	BD Pharmingen™, USA

Anti-human CD40 FITC	5C3	1 μ l/5 x 10 ⁵ cells	BD Pharmingen™, USA
Anti-human CD54 APC	HA58	1 μ l/5 x 10 ⁵ cells	BD Pharmingen™, USA
Anti-human CD80 PE	L307.4	1:10	BD Pharmingen™, USA
Anti-human CD83 APC	HB15e	1:10	BD Pharmingen™, USA
Anti-human CD86 PE-Cy5	2331	1:10	BD Pharmingen™, USA
Anti-human HLA-DR FITC	TU36	1:10	BD Pharmingen™, USA
Anti-human CD19 APC	HIB19	0.5 μ l/5 x 10 ⁶ cells	BD Pharmingen™, USA
Live/Dead APC-Cy7		1:1000	Invitrogen

Table 11: Antibodies used for ELISA

Antibodies	Conjugated	Species	Dilution	Company
Anti-mouse IgA	Purified	mouse	5 μ g / ml (standard)	Sigma-Aldrich
Anti-mouse IgA	Purified	goat	1:200	Sigma-Aldrich
Anti-mouse IgG	Purified	goat	1:200	Sigma-Aldrich
Anti-mouse IgG1	Purified	goat	1:10,000	Sigma-Aldrich
Anti-mouse IgG2a	Purified	goat	1:5,000	SBA (southern biotechnology Associates)
Anti-mouse IgA	Biotinylated	goat	1:5,000	Sigma-Aldrich
Anti-mouse IgG	Biotinylated	goat	1:5,000	Sigma-Aldrich
Anti-mouse IgG1	Biotinylated	goat	1:2,000	SBA
Anti-mouse IgG2a	Biotinylated	goat	1:10.000	SBA

Table 12: Antibodies used for ELISPOT

Antibodies	Conjugated	Species	Dilution	Company
Anti-mouse IFN- γ	Purified	rat	1:200	BD Pharmingen™
Anti-mouse IL-2	Purified	rat	1:200	BD Pharmingen™
Anti-mouse IL-4	Purified	rat	1:200	BD Pharmingen™

Anti-mouse IL-17	Purified	rat	1:250	BD Pharmingen™
Anti-mouse IFN- γ	Biotinylated	rat	1:250	BD Pharmingen™
Anti-mouse IL-2	Biotinylated	rat	1:250	BD Pharmingen™
Anti-mouse IL-4	Biotinylated	rat	1:250	BD Pharmingen™
Anti-mouse IL-17	Biotinylated	rat	1:250	BD Pharmingen™

2.1.8 Adjuvants

Table 13: Adjuvants

Adjuvants	Commentary:
c-di-AMP	Synthesized by Dr. Morr, HZI, dissolved in Ampuwa®, stored at -20°C
c-di-GMP	Synthesized by Dr. Morr, HZI, dissolved in Ampuwa®, stored at -20°C
c-di-IMP	Synthesized by Dr. Morr, HZI, dissolved in Ampuwa®, stored at -20°C

2.1.9 Synthesis of cyclic di-nucleotides

The c-di-GMP and c-di-AMP were synthesized by crystallization according to established protocols [98, 116]. The resulting compound was purified by reversed-phase high-performance liquid chromatography at RT using a Phenomex column (50 by 2 mm, 4 μm , C18) and a gradient of 25 mM triethylammonium formic acid (solvent A [pH 6.8]) to acetonitrile (solvent B) from 0% to 20% solvent B within 20 min at a flow rate of 0.7 ml/min. Subsequently, the sodium form of c-di-GMP was obtained by Dowex- Na^+ ion exchange. The structure of water-soluble c-di-GMP and c-di-AMP was confirmed by ^1H - und ^{13}P -nuclear magnetic resonance and matrix-assisted laser desorption-ionization mass spectrometry. In order to assess if structural related compounds also exert adjuvant activity, bis-(3',5')-cyclic dimeric inosine monophosphate (c-di-IMP) was synthesized out of the parenteral compound c-di-AMP through adenosine-deamination. The final lyophilized compound was stored at -20°C . For the immunization studies, c-di-GMP, c-di-AMP and c-di-IMP were dissolved in sterile water. The cyclic di-nucleotide solution was stable for at least six months at -20°C and two months at 4°C .

2.2 METHODS

2.2.1 Properties of cyclic di-nucleotides

2.2.1.1 HEK Blue™ LPS detection

The HEK-Blue™ cells were thawed under gently agitation in a 37°C water bath and transferred in a sterile tube containing 15 ml of growth medium, DMEM high glucose (4.5 g/L) supplemented with 10% FCS, penicillin-streptomycin and 1 x Normocin™, and spin at 1,500 x g for 5 min. After removing the supernatant containing the cryoprotective agent, cells were re-suspended with 1 ml of the growth medium. The contents of the vial were transferred to a 25 cm² tissue culture flask containing 5 ml of growth medium. The flask was incubated at 37°C (5% CO₂) in a humidified atmosphere until 50–80% confluency was reached. The cells were then trypsinized and cultivated into growth medium supplemented with 1x HEK-Blue™ Selection. Cells were passaged 2 to 3 times a week until 60–80% of confluency was reached and diluted with pre-warmed HEK-Blue™ detection medium at a concentration of 1 x 10⁵ cells/ml. First, to a 96-well sterile flat-bottomed cell culture plate were added 20 µl/well of different dilutions from each sample (1:10, 1:20, 1:40 and 1:80), and 20 µl/well of endotoxin-free water as a negative control. As positive control, 20 µl/well of *E. coli* K12 LPS in serial dilutions of 0.1, 0.3, 1, 10 and 100 ng/ml were added. Afterwards, 200 µl/well of the cell suspension were loaded and plates were incubated at 37°C (5 % CO₂) in a humidified atmosphere for 18 to 24 h. HEK-Blue™-4 cells stably express an optimized alkaline phosphatase gene engineered to be secreted, which is placed under the control of a promoter inducible by several transcription factors such NF-κB. Therefore, the HEK-Blue™ Detection medium turns into a blue colour in the presence of phosphatase activity and can be quantified after the specified incubation time by spectrophotometric measurement at 620-655 nm or with the naked eye by comparing with the positive control which appear in blue and the negative control should be pink or light purple.

2.2.1.2 Nitric Oxide release assay

Activated murine macrophages synthesize nitric oxide (NO), which resolves into nitrate and nitrite. Nitrate is enzymatically transformed into nitrite and can then be detected as an Azo-dye. The amount of NO gives information about the biological activity of the stimulated macrophages. The nitric oxide release assay was performed as previously described [117, 118]. In brief, female C3H/HeJ LPS non-responder mice (Jackson Laboratory, USA) were used at the age of 10 to 18 weeks. Resident murine peritoneal exudate cells were exploited as a source of peritoneal macrophages. To this end, mice were

asphyxiated with CO₂ immediately before injection of about 3 ml of ice-cold PBS with 1% FCS into the peritoneal cavity. After gently massages of the abdomen, the cells were withdrawn, centrifuged at 4°C, and adjust to 2×10^6 cells per ml in DMEM supplemented with 5% FCS, 2 mM L-glutamine and 25 mM 2-mercaptoethanol. Recombinant murine IFN- γ (62.5 U/ml) was added to the cells and 50 μ l of the cell suspension was added to 50 μ l of serial 1:2 diluted adjuvants in medium, in 96-well flat-bottomed microtiter plates. After 45 h incubation at 37°C in a humidified atmosphere with 5% CO₂, 10 μ l of a solution containing nitrite reductase from *Aspergillus* spp (Boehringer # 981 249; 20 U lyophilized) and NADPH trinatrium (Boehringer # 107 824) (equals 8.18:1) was added mixed well and incubated for 10 min. Then 100 μ l/well of Griss reagent, consists of a freshly made 1:1 mixture of 0.1% naphthylethyldiamine- 2-HCl in water and 1% sulphanilamide in 5% H₃PO₄, was added for 10 min. As golden standard R-MALP-2 (approximately 0.2 ng/ml) and LPS (approximately 0.1 μ g/ml) were used (data not shown). The NO-value was analysed with the spectrophotometer plate reader (ELISA Reader) at 550 nm vs. 690 nm.

2.2.2 Evaluation of the adjuvanticity of c-di-AMP

2.2.2.1 Intranasal vaccination with c-di-AMP as adjuvant

For intranasally (i.n.) immunization mice were individually anaesthetized by inhalation of Isofluran® Curamed (Essex Tierarznei, Germany). A maximum of 10 μ l of vaccine formulation per nostril were slowly administered by holding the mouse headlong and waiting until the liquid was completely absorbed. By administrating this small volume it was ensured that the whole dose remains in the nasal mucosa, minimizing the risk that it reaches the lungs or is swallowed. Groups of 3-5 six-weeks-old BALB/c mice were immunized i.n. on days 0, 14 and 28 with 15 μ g of β -Gal or OVA alone or co-administered with 5 μ g/dose of c-di-AMP. Mice of the positive control group were vaccinated using CTB as adjuvant. Animals of the negative control group received PBS or Ampuwa. Fourteen days after the last boost animals were sacrificed by CO₂ inhalation and sera, nasal washes, lung and vaginal lavages, as well as the bone marrow, cervical lymph nodes and spleen were recovered from the mice to analyse humoral and cellular immune responses (Figure 4).

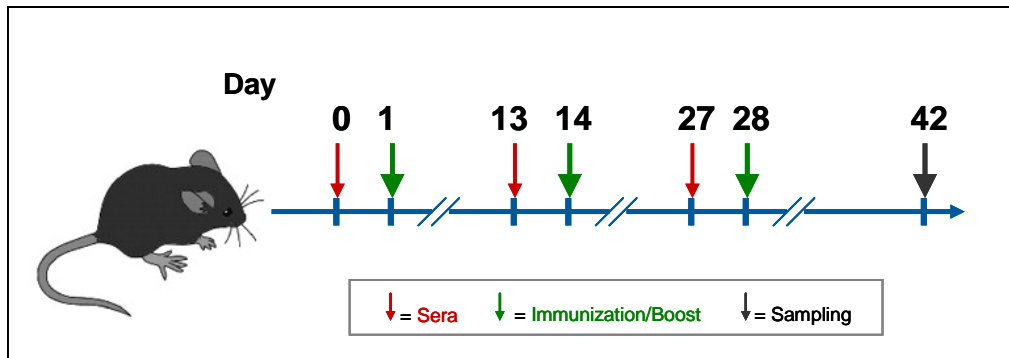


Figure 4: Overview of the immunization protocol

2.2.2.2 Health Control

To control the health of immunized mice and to check for the occurrence of side effects (e.g. fever), the optical appearance, the behaviour and the weight of the individual animals were controlled before first immunization and two, four and six days after the immunization and each booster (day 14 and 28).

2.2.2.3 Sampling of mice

Sera

To monitor humoral immune responses, the presence of antigen-specific IgG in sera of immunized mice was analysed. During an immunization experiment individual blood samples were taken before the first immunization (day -1), the second immunization (1st boost, day 12), the third immunization (2nd boost, day 26) and 14 days after the last vaccination dose. Blood samples were taken via the retro-orbital plexus by using 40 µl glass capillaries. Serum was obtained by allowing the blood to coagulate during 1 h (incubation at 37°C), followed by 30 min incubation at 4°C. To separate the serum from other blood components, the tubes were centrifuged at 7,000 x g in a table centrifuge for 10 min at room temperature (RT). Then, supernatants were carefully removed and stored at -20°C (until ELISA).

Euthanasia of mice

To sample spleens, NALT, lymph nodes (LN), bone marrow derived DCs and vaginal lavages the mice were sacrificed by cervical dislocation. When the mucosal immune responses of the lungs or noses were examined, mice were euthanized by CO₂ inhalation instead of cervical dislocation in order to prevent blood from flooding to the lungs and mixing with the secretions of the mucosal membranes.

Collection of Spleen

To prevent contamination the mouse was soaked with 70% ethanol. The abdomen was carefully disembowelled and by using tweezers and scissors the spleen was removed and stored in RPMI complete on ice. Spleens of immunized mice were pooled for each group.

Nasal Lavage

To detect secretory IgA in the nasal cavities following i.n. immunization, the lower jaw was cut and a 200 μ l tip was inserted into the posterior opening of the nasopharynx. Nasal cavities were then flushed twice with 200 μ l of lavage buffer (5% FCS/1xPBS), which was collected in a 1.5 ml tube with 10 μ l of 40 mM PMSF. The collected probes were centrifuged at 7,000 x g in a table centrifuge for 10 min at RT. The supernatant was carefully removed and stored at -20°C until the IgA ELISA was performed.

Lung Lavage

To analyse the stimulation of secretory IgA in the lung after immunization, individual lung lavages of each mouse were obtained. The neck skin and muscles were carefully removed with tweezers and a small cut was set in the middle of the trachea between two rings without intersecting the whole trachea using scissors. A catheter of 1 mm diameter was carefully inserted into the lung. Via a 1 ml syringe 1,000 μ l of lavage buffer (5% FCS/1xPBS) were injected and the ribcage was massaged to get the secretory components. Then, the buffer was withdrawn and collected in a 1.5 ml tube with 10 μ l of 40 mM PMSF. The collected probes were centrifuged at 7,000 x g in a table centrifuge for 10 min at RT. The supernatant was carefully removed and stored at -20°C until the IgA ELISA was performed.

Vaginal Lavage

To investigate secretory IgA production after immunization in the genital tract, individual vaginal lavages were taken from each mouse. To this end, the mucosa of the vagina was flushed two times with 500 μ l of lavage buffer (5% FCS/1xPBS) by carefully inserting a 1,000 μ l tip into the vagina. After pipetting several times up and down, the lavage probes were collected in a 1.5 ml tube with 10 μ l of 40 mM PMSF for preventing the degradation of proteins. Then, the probes were centrifuged at 7,000 x g for 10 minutes at RT to remove the debris. Finally, the supernatant was carefully taken off and collected in a 1.5 ml tube filled with 10 μ l of 40 mM PMSF and stored at -20°C for determination of IgA by ELISA.

2.2.2.4 Preparation of single cell suspension of spleen, cLN, NALT and BM

Spleen

To obtain a single cell suspension of the spleens and cervical lymph nodes (cLN), the organs were mechanically dissociated in RPMI complete by gently pressing them through a 100 μ m cell mesh using the rubber cap of a 2 ml syringe plunger. The cell suspension was transferred to a 50 ml Falcon tube and centrifuged at 300 x g for 10 min at RT. For the depletion of the erythrocytes the pellet was re-suspended in ACK lysis buffer (3 ml/spleen). After 60 seconds the tube was filled with RPMI complete to stop the lysis. The cell suspension was centrifuged as described above, the pellet was re-suspended in RPMI complete (4 ml/spleen) and filtered through a 100 μ m mesh to remove the released DNA fragments and cell debris. The number of cells was then determined by using the Neubauer cell counter chamber or the cell counter Z2 from Beckman-Coulter and adjusted to 5×10^6 cells/ml. Cells which were not immediately needed, were re-suspended in freezing medium (10% DMSO in FCS) at a concentration of 10^8 cells/ml and transferred into cryotubes. To achieve the recommended cooling of 1°C/min the cells were retained for 24 h at -70°C in Nalgene® Cryoboxes boxes filled with isopropanol. After 24 h the cells were stored at -196°C in the liquid phase of a nitrogen tank until further usage.

NALT

For the isolation of APCs in the NALT 24 h after intranasal administration of c-di-AMP, the lower jaw and tongues were removed, then the upper palate was removed with scalpel and tweezers, and NALT were taken and transferred onto a Petri dish containing RPMI-complete medium. Cells were scratched from the nasal cavity walls and single-cell suspensions were prepared by mincing the tissue through a 100 μ m nylon mesh. Cells were washed twice with RPMI complete medium at 300 x g, for 10 min at RT and then prepared for flow cytometry staining, as described above.

cLN

Following i.n. immunization cervical draining lymph nodes were collected in RPMI complete medium and prepared in the same way as described above for splenocytes. Afterwards, cells were used for APC flow cytometric analysis.

2.2.2.5 *Detection of cell number*

The cell number can be determined using either the Neubauer cell counter chamber (haemocytometer) or the electronic cell counter Z2 (Beckman-Coulter). For counting cells in the Neubauer counting cell chamber, the cell suspensions are diluted in Trypan blue solution, added into the haemocytometer chamber and enumerated under the light microscope. Trypan Blue is excluded from living cells, whereas dead cells showed an enhanced uptake of the blue coloured dye. Depending on the concentration, the cell suspension was mixed with Trypan blue solution to reach a final dilution (y), which allows counting of the cells. The cells were filled into the chamber of the haemocytometer and the non-stained, i.e. viable, and dead cell cells were enumerated by using a microscope. After counting the cells in the 4 big squares, each consisting of sixteen small quadrates, the cell number per ml was determined by the following formula:

$$\text{Total cell number} = (\text{counted cells} / 4 \text{ squares}) \times \text{dilution (y)} \times 10^4$$

The other possibility to determine the cell number is the usage of the electronic cell counter Z2. The method of sizing and counting particles with the electronic cell counter is based on measurable changes in electrical resistance produced by non-conductive particles suspended in an electrolyte. In the sensing zone, i.e. a small opening between electrodes, each passing particle displaces its own volume of electrolyte which is measured as a voltage pulse; the height of each pulse being proportional to the volume of the particle. The Beckman-Coulter counter reports both count and concentration and provides the possibility to display the size distribution of the cell population. In comparison to the Neubauer counting cell chamber, dead and living cells cannot be distinguished in one step. To determine the cell number using the electronic counter, the cell suspension was diluted 1:500 in Isotone II, a near physiological saline solution. Spleen cells were measured at a size between 5 µm and 16 µm of diameter by using the Multi32 Coulter Z2® AccuComp® v3.01 software (Beckman-Coulter).

2.2.2.6 *Detection of antigen-specific antibodies by ELISA*

Antigen-specific IgG

β-Gal- and OVA-specific antibodies were determined in serum samples by ELISA using microtitre plates coated with 100 µl/well of the respective antigen (5 µg/ml in 0.05 M carbonate buffer (pH 9.6), as previously described [119]. After overnight incubation at 4°C, plates were blocked with 3% BSA in PBS for 1 h at 37 °C. Serial 2-fold dilutions of sera in 3%

BSA/1xPBS were added (100 μ l/well), and plates were further incubated for 2 h at 37 °C. After six washes with 1% BSA/1xPBS/0.05% Tween 20, secondary antibodies were added (see Table 11), such as biotinylated γ -chain-specific goat anti-mouse IgG or for the determination of the different IgG subclasses, biotinylated goat anti-mouse IgG1 and IgG2a (Sigma, USA) for BALB/c mice. Subsequently, the samples were incubated at 37°C for 2 h [119]. After six washing steps, 100 μ l of peroxidase-conjugated streptavidine (BD Pharmingen, USA) was added to each well, and plates were incubated at RT for 1 h. After another six washes, reactions were developed using ABTS in 0.1 M citrate–phosphate buffer (pH 4.35) containing 0.01% H₂O₂. Endpoint titres were expressed as absolute values of the last dilution which gave an optical density at 405 nm of 2 times above the values of the negative controls after 15 and 30 min of incubation.

Antigen-specific IgM, IgA and SC in mucosal secretions

The amount of antigen-specific secretory antibodies IgM, IgA and SC present in the lavage samples of nasal cavity, lung and vagina was determined by ELISA, as previously described [119]. Briefly, 96-well microtitre plates were coated overnight at 4°C with 100 μ l/well of β -Gal or OVA at a concentration of 2 μ g/ml diluted in carbonate buffer. After blocking with 3% BSA/PBS for 1 h at 37 °C, the plates were washed and further incubated with 2-fold serial diluted lavages samples (starting with 1:4) for 1 h at 37°C. After four washes, anti-mouse IgA or IgM biotinylated detection antibodies (Sigma, USA) were added in each well. The plates were further incubated for 1 h at 37°C. After the plates were washed, peroxidase-conjugated streptavidine (BD Pharmingen, USA) was added and the plates were incubated at RT for 1 h. The plates were washed and reactions were developed using ABTS in 0.1 M citrate–phosphate buffer (pH 4.35) containing 0.01% H₂O₂. For the detection of the SC, plates coated with β -Gal were blocked with 5% of normal donkey serum (v/v) for 2 h. Serial dilutions of the lavage samples were incubated (1 h) and subsequently washed. The antibodies containing the SC were detected by 1 h incubation with a 1:200 dilution of a goat anti-pIgR (R&D Systems; USA). For the detection, plates were further incubated with a 1:10,000 dilution of HRP-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, USA) followed by development with ABTS diluted in 0.1M citrate–phosphate buffer (pH 4.35) containing 0.01% H₂O₂. Results were expressed as endpoint titres of the last dilution which gave an optical density at 405 nm of two times above the values of the blank after 30 min of incubation.

2.2.2.7 Enzym-Linked Immunospot Assay (ELISPOT)

ELISPOT assays were performed using flat bottomed 96-well plates with a 0.45 μm hydrophobic High Protein Binding Immobilin-P-Membran. The plates were coated with 100 μl /well of the corresponding capture antibody (anti-IFN- γ , anti-IL-2, anti-IL-4 or anti-IL-17), diluted 1:200 in PBS and stored overnight at 4°C. To remove unbound capture antibody, the plate was washed once with 200 μl /well of blocking solution (RPMI complete medium). Then, the unspecific binding sites were saturated by incubating with 200 μl /well of blocking solution for 2 h at RT. Afterwards, 200 μl /well corresponding to 1×10^6 cells/well of single spleen cell suspensions were added in triplicates to the plate. To analyse IFN- γ secretion, the spleen cells were re-stimulated by adding 50 μl /well of the CD8⁺-specific β -Gal peptide diluted in RPMI complete medium to reach a final concentration of 5 $\mu\text{g}/\text{ml}$. The cells used for detection IL-2, IL-4 or IL-17 secretion were co-cultured with 5 $\mu\text{g}/\text{ml}$ of β -Gal. As negative control, cells were cultured in RPMI complete medium without stimulants. As positive control, ConA diluted in RPMI complete medium (final concentration 5 $\mu\text{g}/\text{ml}$) was used to stimulate 1×10^6 cells/well. The plates were incubated at 37°C and 5% CO₂ for 24 or 48 h to detect IFN- γ or IL-2, IL-4 and IL-17 secretion, respectively. After the incubation, the cell suspension was aspirated and the plates were washed twice with deionized water including a soaking for 5 min followed by three washes using 200 μl /well of wash buffer (0.05 % Tween-20/PBS). To detect the captured cytokines, 100 μl /well of the corresponding biotinylated detection antibody diluted 1:250 in dilution buffer (10% FCS/PBS) was added and incubated for 2 h at RT. After washing three times with 200 μl /well of wash buffer, 100 μl /well of avidin HRP diluted 1:100 in dilution buffer were incubated for 1 h at RT. The plates were then washed four times with 200 μl /well of wash buffer and subsequently twice with PBS. Spots were developed for 5 to 60 min using 100 μl /well of substrate solution (333.3 μl of AEC stock solution + 10 ml of 0.1 mM acetate solution + 5 μl H₂O₂). The reaction was stopped by washing the plate with deionized water. After drying the plates for 2 h at RT in the dark, the plates were scanned with an ImmunoSpot series 3A analyzer and spots were counted by using the ImmunoSpot image analyzer software (version 3.2; Cellular Technology, Ltd.). The results were expressed as spot forming units (SPU) for 1×10^6 spleen cells/well. The spots produced by the non re-stimulated cells served as background and were subtracted from the spots produced by the re-stimulated cells. Antibodies used for ELISPOT are listed in Table 12.

2.2.2.8 Determination of lymphoid-mediated cytotoxicity in vivo

To investigate the influence of c-di-AMP on the stimulation of antigen-specific CTLs, splenocytes obtained from naïve C57BL/6 mice were washed twice with serum-free RPMI

medium at 200 x g for 10 min at RT and split into two equal proportions, each at a concentration of 1×10^7 cells/ml. One aliquot was labelled with a high concentration of CFSE (1 μ M), whereas the other one was labelled with a low concentrated CFSE (0.1 μ M). The labelled splenocytes were incubated for 7 min at 37°C and 5% CO₂ in the dark. The labelling was interrupted by adding an equal volume of medium containing 5% FCS for 10 min at 37°C and 5% CO₂. Afterwards, the cells were washed twice with RPMI complete medium and the high CFSE labelled spleen cells were pulsed with the OVA peptide (amino acids 257 to 264) at a concentration of 15 μ g/ml for 1 h at 37°C and 5% CO₂. The low CFSE labelled cells were incubated without peptide stimulation. After 1 h cells were washed twice and an equal number of cells deriving from each type of labelling were mixed. A total of 2×10^7 cells were injected intravenously into mice immunized by parenteral or mucosal route with OVA (50 μ g/mouse) co-administered with 5 μ g c-di-AMP two weeks after last vaccination (day 1, 14, 28). After 16 h, spleens were collected and analysed by flow cytometry. Specific lysis was determined by the decrease of CFSE of the peptide pulsed population in comparison with the non-stimulated cells. The following formula was used to calculate the percentage of OVA-specific lysis:

$$\frac{100 - (\% \text{ CFSE}^{\text{high}} \text{ in immunized mice} / \% \text{ CFSE}^{\text{low}} \text{ in immunized mice})}{(\% \text{ CFSE}^{\text{high}} \text{ in control mice} / \% \text{ CFSE}^{\text{low}} \text{ in control mice})}$$

2.2.2.9 T cell proliferation assay

The ability of T cells to proliferate after efficient stimulation can be determined by measuring the uptake of radioactive [³H]-thymidine, which is building in the DNA. To determine the proliferative capacity of spleen cells, single spleen cell suspensions of immunized mice were prepared as described above, adjusted to 5×10^6 per ml in RPMI complete medium and 100 μ l/well were seeded in a 96-well sterile flat-bottom cell culture plate. For the re-stimulation of spleen cells, 100 μ l/well of β -Gal diluted in RPMI-complete medium were added in quadruplicates (final concentration of 40, 20, 10, 5 and 1 μ g/ml). As positive control, the mitogen concavalin A (ConA) was used at the final concentration of 5 μ g/ml, whereas the negative controls received medium alone. The plates were incubated at 37°C and 5% CO₂ in a humidified atmosphere. After four days of incubation, splenocytes were pulsed with 50 μ l/well of [³H]-thymidine diluted in RPMI-complete medium (1 μ Ci/well) during the final 16-18 h of culture. Then, cells were harvested on glass fibre filters by using a cell harvester (InoTech). After drying the filter in a microwave, the scintillator sheet was melt on the filter. This transfer of [³H] thymidine into the wax layer, which constitutes a kind of

scintillation fluid, allows the final measuring of the radioactivity using the gamma-scintillation counter (1450 Microbeta, Wallach Trilux). Results were expressed as the arithmetic mean of [^3H]-thymidine uptake in counts per minute (cpm) or as stimulation indexes, being the ratio of [^3H]-thymidine uptake of stimulated versus non-stimulated samples.

2.2.2.10 Stimulation of cytokine secreting cells

Cytokines secreted by re-stimulated immune cells exert effector functions on immune cells, thereby affecting the outcome of both cellular and humoral immune responses. This depends on the type of cytokines which are produced by the specific Th subset (e.g. Th1, Th2). Thus, to investigate the type and amount of cytokines secreted from re-stimulated spleen cells of immunized mice, spleen single cell suspensions were *in vitro* re-stimulated using different β -Gal concentrations according to the T cell proliferation assay. Briefly, 5×10^5 spleen cells were incubated in quadruplicates, with or without β -Gal (40, 20, 10, 5 and 1 $\mu\text{g/ml}$ final concentration) in a total volume of 200 μl for 96 h at 37°C and 5% CO_2 . Positive control cells were stimulated with ConA (5 $\mu\text{g/ml}$). After centrifugation for 5 min at $300 \times g$ at RT, the supernatant was carefully aspirated and stored at -70°C until the secreted cytokines were analysed by different cytokine array assays. For quantification, the contents of IL-6, IL-10, IL-17 and IFN- γ in the collected supernatants were determined using a Mouse Th1/Th2 10plex FlowCytomix cytokine array according to the manufacturer's instructions (eBioscience, Bender MedSystems®, USA). Supplied standards were used to generate standard curves.

2.2.3 Examination of the immune effector functions of c-di-AMP on murine immune cells

2.2.3.1 Generation of bone-marrow derived dendritic cells (BMDCs)

Ten to twelve weeks old BALB/c mice were killed and carefully sprayed with ethanol 70% to prevent contaminations. After removing the skin and muscles using a pair of scissors, the hind legs were disconnected from the body in front of the hip. The feet were cut off and the intact femurs and tibias were put in a Petri dish with ethanol 70% for 2 to 5 min for disinfection. Then, the bones were transferred in a Petri dish with DC medium and stored on ice. To obtain the bone marrow, the bones were flushed with medium using a 1 ml syringe and a 26G $\times \frac{1}{2}$ needle and collected in a tube. The end of the bones and the flushed bones were disrupted and crushed with a strong pair of scissors. The pellet was re-suspended in ACK buffer (5 ml for the bone marrow of 10 mice) to remove the erythrocytes. The lysis was stopped after 2 min by adding 20 ml of DC medium and after centrifugation for 10 min at 300

x g at RT the cells were re-suspended in DC medium. Finally the cells were filtrated through a 100 μ m mesh to remove released DNA fragments and pieces of fat. The cells were adjusted to 1×10^7 cells/ml in DC medium, seeded onto a 10 cm diameter cell culture dish with 10 ml/dish, and incubated at 37°C (5 % CO₂) in a humidified atmosphere for 1-2 h to remove adherent cells like macrophages. The non-adherent cells were collected and centrifuged for 10 min at 300 x g at RT. The pellet was re-suspended in DC medium supplemented with either 5 ng/ml cytokine GM-CSF or 100 ng/ml FMS-like tyrosine kinase 3 ligand (Flt3L), for obtaining differentiated DCs, respectively. The cells were adjusted to a concentration of 1×10^6 cells/ml, seeded in 6-well-plates at 5 ml/well and incubated for at 37°C (5% CO₂) in a humidified atmosphere. After 3 days, 1 ml of the supernatant was carefully aspirated from each well and replaced with fresh GM-CSF supplemented DC medium. The GM-CSF stimulated DCs were used on day 5 or 6, whereas the Flt3L stimulated DCs were used on day 9 or 10 for *in vitro* stimulation with adjuvants.

2.2.3.2 Generation of murine bone-marrow derived macrophages (MΦs)

To generate bone marrow-derived primary macrophages (MΦs) from C57BL/6 or BALB/c mice, femur and tibia of mice were flushed with medium, as described above. Erythrocytes were lysed by adding ACK buffer. Finally the cells were filtrated through a 100 μ m mesh to remove released DNA fragments and pieces of fat. The cells were adjusted to 1×10^7 cells/ml in MΦ medium supplemented with M-CSF, seeded onto a 10 cm diameter cell culture dish with 10 ml/dish, and incubated at 37°C (5 % CO₂) in a humidified atmosphere. Every 2-3 days the cell culture dishes were washed twice with cold PBS and fresh MΦ media was added. After 7-9 days the MΦs were detached with a rubber policeman by careful scratching the surface of the Petri dishes, and used for *in vitro* stimulation with adjuvants.

2.2.3.3 In vitro stimulation of DCs and MΦs with c-di-AMP

To analyse the effect of adjuvants on the maturation and activation of murine DCs and MΦs, cells were stimulated *in vitro* with c-di-AMP or LPS. From each well, 1 ml of supernatant was carefully aspirated and substituted with fresh DC or MΦ medium supplemented with GM-CSF or M-CSF and the corresponding stimulants. The adjuvant was used at final concentration of 1 μ g/ml for DCs and MΦs. As positive control, LPS, a well-known stimulator of DCs and MΦs [120], was used at a final concentration of 1 μ g/ml. Non-stimulated DCs or MΦs were used as negative control. The cells were incubated for 24 h at 37°C (5% CO₂) in a humidified atmosphere, then, they were collected in a tube and centrifuged at 300 x g for 10 min at RT. To investigate adjuvant induced changes in murine

DC and MΦ maturation and activation, the expression of the phenotypic markers CD40, CD54, CD80, CD86, and MHC class I and II (Table 8) was evaluated by flow cytometry.

2.2.3.4 Isolation and stimulation of murine B cells

To analyse the effect of c-di-AMP on the activation of murine B cells *in vitro*, splenocytes taken from 6-8 weeks old BALB/c mice were isolated as described before. For the depletion of the erythrocytes the pellet was re-suspended in ACK lysis buffer (3 ml/spleen). After 60 seconds the tube was filled with RPMI complete to stop the lysis. The cell suspension was centrifuged as described above, the pellet was re-suspended in RPMI complete (4 ml/spleen) and filtered through a 100 µm mesh to remove the released DNA fragments and cell debris. The number of cells was determined by using the cell counter Z2 from Beckman-Coulter. Then, cells were stained with for the B cell marker CD19 for 30 min at 4°C and afterwards washed with 1x PBS and adjusted to 2×10^7 cells/ml and sorted for CD19⁺ cells (purity of 98%). To investigate the activation of B cells induced by the adjuvant, stimulated cells were stained for the markers CD40, CD80, CD86, and MHC class II (Table 8) and further analysed by flow cytometry.

2.2.3.5 B cell proliferation assay

To determine the proliferative capacity of B cells, CD19 sorted B cells were stimulated with the adjuvants as described above, adjusted to 5×10^6 per ml in RPMI complete medium and 100 µl/well were seeded in a 96-well sterile flat-bottom cell culture plate. As positive control, ConA was used at the final concentration of 5 µg/ml, whereas the negative controls received medium alone. The plates were incubated at 37°C and 5% CO₂ in a humidified atmosphere. After four days of incubation, splenocytes were pulsed with 50 µl/well of [³H] thymidine diluted in RPMI-complete medium (1 µCi/well) during the final 16-18 h of culture. Then, cells were harvested on glass fibre filters by using a cell harvester (InoTech). After drying the filter in a microwave, the scintillator sheet was melt on the filter. This transfer of [³H] thymidine into the wax layer, which constitutes the scintillation media, allows the final measuring of the radioactivity using the gamma-scintillation counter (1450 Microbeta, Wallach Trilux). Results were expressed as the arithmetic mean of [³H] thymidine uptake in counts per minute (cpm) or as stimulation indexes, being the ratio of [³H] thymidine uptake of stimulated versus non-stimulated samples.

2.2.3.6 *Measurement of cellular proliferation of antigen specific murine CD4⁺ and CD8⁺ T cells*

Differentiated murine DCs were incubated with 10 µg/ml of LPS-free OVA (EndoGrade® Ovalbumin, #321001, Hyglos, Germany) and 1 µg/ml c-di-AMP or LPS. The following day, CD8⁺ T cells from spleens of OT-I mice, which have a transgenic T cell receptor specific for the OVA immunodominant peptide (aa 257-264, SIINFEKL) presented in the context of the MHC class I H-2Kb, or CD4⁺ T cells derived from spleen of OT-II mice expressing the TCR specific for OVA (aa 323–339, ISQAVHAAHAEINEAGR) presented in the MHC class II molecule I-Ab were isolated. Afterwards, the spleen cells were negatively selected by magnetic bead separation (Miltenyi Biotec, Germany), stained with 10 µM CFSE (Molecular Probes, USA) in PBS for 5 min and co-cultured with antigen stimulated DCs for 4 days. The percentages of vital CFSE⁺ labelled CD8⁺ and CD4⁺ cells were then determined by flow cytometry on a BD LSRII and analyzed using the BD FACSDiva software.

2.2.3.7 *Flow cytometric analysis of murine cells*

Surface staining

To investigate cell surface markers by flow cytometry, the cells were stained with fluorescence labelled antibodies. Every step was performed on ice to prevent the internalization of surface molecules or the degradation of cells. The different washing steps were important to stabilize the physiologic milieu of the cells. The cell suspensions were adjusted to 5 x 10⁶ cells/ml and added in 100 µl/well to a 96-well round bottom microtiter plate. Cells were washed by centrifugation at 200 x g for 5 min at 4°C. Afterwards, supernatants were discarded and cell pellets were re-suspended in 50 µl of FACS buffer containing FcR-block diluted 1:2,000. Plates were incubated for 20-30 min on ice. Subsequently, cells were centrifuged and after discarding the supernatant, fluorochrome-labelled antibodies diluted in FACS buffer at the indicated concentrations were added to the appropriate wells in a final volume of 50 µl/well (Table 8). In addition, to each staining combination a fixable live/dead cell marker was added to exclude dead cells. After 30 min of incubation on ice in the dark, cells were washed with 100 µl/well of FACS buffer and the plate was centrifuged for 10 min at 200 x g. To fix and stabilize the cells, cell pellets were re-suspended in 200 µl PBS containing 4% PFA, transferred to a FACS tube and stored on ice in the dark until analysis by FACS LSRII (BD Bioscience, USA). To prevent false positive signals when dyes were used with overlapping emission spectra, single staining for each used antibody was performed for later compensation purposes during data analysis.

Intracellular staining

Following surface staining, cells were permeabilized with 100 μ l/well of Cytofix/Cytoperm (BD Bioscience), re-suspended and incubated for 10 min at 4°C. Afterwards, cells were washed twice with 100 μ l/well Perm wash buffer (diluted 1:10 in water) and centrifuged at 200 x g for 5 min at 4°C. Then, the supernatants were discarded and cells were stained with the appropriate antibodies (Table 9) diluted in Perm wash buffer at a final volume of 50 μ l/well. After 20 min of incubation at 4°C in the dark, cells were washed twice with Perm wash buffer, re-suspended in 200 μ l PBS and stored at 4°C in the dark until analysis.

2.2.3.8 Evaluation of APC activation ex vivo

To study the *in vivo* effector functions on innate immune cells of the adjuvant, Balb/c mice received c-di-AMP at a concentration of 5 μ g per mouse by i.n. route. Control mice were treated with either PBS or MALP-2 (0.5 μ g/dose) as well-known TLR2/6 agonist [118]. After 24 h cells from spleen, NALT and cLN were collected and prepared for each mouse separately, as described above. Afterwards, the obtained cell suspensions were analysed in terms of surface markers predicting for APC and DCs subset activation and functionality by flow cytometry. The used antibodies are listed in Table 8. The staining procedures were performed as described above.

2.2.3.9 Electron microscopy analysis of Nanogold-labelled c-di-AMP

The Mono-Sulfo-NHS-NANOGOLD® (1.4 nm mean diameter; Nanoprobes, Stony Brook, NY) reagent (6 nmol) was dissolved in 200 μ l deionized water by extensive vortexing. A 20-fold excess of the adjuvant c-di-AMP were used for labelling and incubated 12–18 h (overnight). The Nanogold-c-di-AMP conjugate was then given to 6 days old bone-marrow derived dendritic cells (BMDCs) and incubated for different time points (0.5 min, 5 min, 30 min, 2 h, and 6 h) at a humidified atmosphere. Cells were fixed in 1% (v/v) glutaraldehyde (GA) for 15 min at RT. Afterward, they were centrifuged for 5 min at 300 x g, the supernatant was discarded and cells were re-suspended in 1 ml of 1 x PBS. The washing step was repeated two times.

Fixation and embedding

Samples from BMDCs were immediately fixed at RT in 2.5% (v/v) GA in 20 mmol L-1 HEPES buffer (pH 7.1) for 30 min. Cells were stored until embedment for several days at 4°C. For ultrastructural analysis, cells were immobilized in 2% (w/v) agar. Cells were

dehydrated on ice in an ethanol series, stained with 1% (w/v) uranyl acetate in 70% (v/v) ethanol and finally infiltrated with epoxy resin [121]. Cells were polymerized at 70°C for 8 h. Ultrathin sections (70 nm) were cut with a diamond knife using an ultramicrotome (Leica, Wien, Austria), picked with Formvar-coated 300 mesh copper grids and post-stained with uranyl acetate and lead citrate, as described previously [122].

Silver Enhancement

Cells were rinsed with deionized water (2 X 5 min) following 0.02 M sodium citrate buffer, pH 7.0 (3x 5 min). Then, the grid was floated with specimen on freshly mixed developer for 1-8 min, or as directed in the instructions for the silver reagent. More or less time can be used to control particle size. With HQ Silver, a development time of 6 min gives 15-40 nm round particles. The cells were rinse again with deionized water (3x 1 min) and mounted. Sections were viewed in a JEOL 1010 or a JEOL 1200 electron microscope (JEOL; Tokyo, Japan), and images were recorded on Kodak 4489 sheet films (Kodak; Rochester, NY).

2.2.3.10 Immunofluorescence Confocal Microscopy of 2'-Fluo-AHC-c-di-AMP

Indirect Immunofluorescence

BMDCs analysed by confocal microscopy were putted on collagen-coated coverslips (BD BioCoat, BD Bioscience, USA) in 24 well plates (Nunc, Denmark). DCs were stimulated with 1.5 μ M 2'-Fluo-AHC-c-di-AMP (Biolog) for 2 h. After incubation, cells were carefully washed with pre-warmed PBS and fixed by adding 3% PFA for 10 min at RT. Fixed cells on coverslips were quenched for 15 min in 50 mM glycine in PBS. Subsequently, cells were permeabilized with 0.05% saponin in PBS containing 1% BSA, and then they were incubated 1 h with the primary and 30 min with the secondary Abs. Finally, the nucleus staining was performed using 1 μ g/ml DNA stain of Hoechst 33258. After staining, the coverslips were mounted on slides using aqueous mounting medium (Dako Cytomation, Denmark) and analysed by confocal microscopy (Leica SP5).

2.2.3.11 Evaluation of type I IFN induction in reporter mice

To study the effect of c-di-AMP on the induction of IFN- β genes, global reporter albino C57BL/6 IFN- $\beta^{\Delta\beta-luc}$ mice bred at HZI were used. Conditional reporter mice IFN- $\beta^{\text{flox}\beta-luc}$ were generated using C57BL/6 ES-cells (Bruce4). To replace the IFN- β CDS by luciferase in germ line (IFN- $\beta^{\Delta\beta-luc}$), IFN- $\beta^{\text{flox}\beta-luc}$ mice were crossed with K14cre mice [123]. To obtained conditional reporter mice expressing the luciferase gene under the control of on B cells, T cells, DCs and macrophages, IFN- $\beta^{\text{flox}\beta-luc}$ were crossed to CD19cre [124], CD4cre [125],

CD11ccre [126] and LysMcre [127] mice, respectively. The conditional reporter mice and IFNAR^{-/-} mice received c-di-AMP by i.n. route at a concentration of 5 µg per mouse. IFN-β gene induction was analysed by measuring luciferase by *in vivo* imaging. To this end, mice were injected intravenously with 150 mg/kg of D-luciferin firefly (Synchem) dissolved in PBS at time points 0, 3, 6, 12 and 24 h after administration of c-di-AMP. Afterwards, mice were anesthetized with Isofluran® Curamed and monitored using the IVIS 200 imaging system (CaliperLS). Photon flux was quantified with the Live Imaging 3.2 Software (CaliperLS).

2.2.3.12 Analysis of Mitogen-Activated Protein Kinases (MAPK) of J774 cells

The Human Phosphor-MAPK Array Kit (The Proteome Profiler™ Array from R&D, #ARY002) is a rapid, sensitive test, which can detect the relative levels of phosphorylation of nine MAPKs and other nine serine/threonine kinase. Several of the human antibodies of this kit are cross-reactive with mouse. Therefore, this kit was used to assess the protein kinase activation after c-di-AMP treatment of J774A.1 cells, according to the recommendations of the manufactures (see below).

Cell lysates

Cells were rinsed with PBS, making sure that any remaining PBS was removed before addition of the lysis buffer. Cells were then suspended at a concentration of 1×10^7 cells/ml in Lysis Buffer 6, by pipetting up and down and they the resulting lysates were subsequent gently rocked at 2-8° C for 30 min. Then, the samples were centrifuged in a microcentrifuge at 14,000 x g for 5 min, and the supernatant fluids were transferred into a clean test tube. Protein concentrations were determined using the Pierce® BCA Protein assay kit. For incubation with the Human Phospho-MAPK Array, a quantity of lysate similar to that used for immunoprecipitation or Western blot (100 - 300 g) was used in a maximum lysate volume of 250 µl/array. Lysates were aliquoted and immediately stored at ≤ -70°C.

Assay procedure

Into each well of the 4-Well Multi-dish 1.5 mL of the Array Buffer 1 were pipetted. With a flat-tip tweezers, each array was removed from the protective sheets and one array into each well of the 4-Well Multi-dish was placed and incubated for 1 h on a rocking platform shaker. In a separate tube, the desired quantity of lysate to 1.25 mL of Array Buffer 1 was added. A final volume of 1.5 mL with Lysis Buffer 6 as necessary was adjusted. The maximum allowable lysate volume is 250 µl/array. The Array Buffer 1 from the 4-Well Multi-dish was removed and the diluted lysates was added and incubated overnight at 2-8°C on a rocking platform shaker, covered with the lid. Each array was removed carefully and place

into individual plastic containers with 20 mL of 1x Wash Buffer. The 4-Well Multi-dish was rinsed with deionized or distilled water and dry thoroughly. Each array was washed with 1x Wash Buffer by soaking for 10 min on a rocking platform shaker. This was repeated two times for a total of three washes. For each array, 15 μ L of Detection Antibody Cocktail Concentrate were diluted to 1.5 mL with 1x Array Buffer 2/3 and 1.5 mL/well of diluted Detection Antibody Cocktail were pipetted into the 4-Well Multi-dish. Then the array was returned to the 4-Well Multi-dish containing the diluted Antibody Cocktail Concentrate covered with the lid, and incubated for 2 h at RT on a rocking platform shaker. The washing described above was repeated and the Streptavidin-HRP was diluted 1:2,000 in 1x Array Buffer 2/3 and 1.5 mL were pipetted into each well of the 4-Well Multi-dish. After an incubation time of 30 min at RT on a rocking platform shaker each array was washed again, as described above. Carefully, each array from the wash container was removed and the excess Wash Buffer was drain from the array. Each array was exposed to chemiluminescent reagents by the use of luminol as a chemiluminescent agent, covered with plastic wrap and exposed to X-ray film for 5-10 min. The developed x-ray film is then quantified by scanning the film on transmission-mode scanner and the array analysed by Multi-Analyst (Bio-Rad Laboratories, Inc.).

2.2.4 Characterization of the effector functions of c-di-AMP on human cells

2.2.4.1 Generation of human monocytes-derived DCs and M Φ s

DCs were prepared from purified peripheral blood monocytes from healthy donors by density gradient centrifugation using Ficoll-Paque (GE Healthcare) and the CD14⁺ cells were isolated by depletion with magnetic beads (Monocyte isolation kit II, Miltenyi Biotec, Germany). For DC differentiation the CD14-enriched monocytes were cultured (1×10^6 cells/ml) in complete culture medium RPMI 1640 with the addition of 25 ng/ml human rGM-CSF and 25 ng/ml human recombinant IL-4 (Invitrogen, CA) in a humidified atmosphere containing 5% CO₂ at 37°C. The medium was changed after 3 days, and on day 6 cells were used for stimulation with or without cyclic di-AMP for 24 h at 37°C. For M Φ differentiation the CD14-enriched monocytes were cultured (1×10^6 cells/ml) in complete culture medium RPMI 1640 with the addition of 50 ng/ml human M-CSF (Invitrogen, CA) in a humidified atmosphere containing 5% CO₂ at 37°C. On day 4, 1 ml of the medium was replaced with 1 ml of fresh medium containing 50 ng of human M-CSF. On day 7, non-adherent cells were washed out and adherent cells were used as macrophages.

2.2.4.2 Human allogeneic proliferation assay

Immature DCs and c-di-AMP-treated DCs were washed three times, diluted in fresh complete medium, and used as allogenic stimulators. Cells were seeded in 96-well round-bottom culture plates with 10,000 DCs/well, and were mixed along with freshly purified CD4⁺ or CD8⁺ T cells (100,000/well). After 3 days of incubation, cells were pulsed with 1 µCi of [3H] thymidine/well for 18 h and were harvested on filter paper. Proliferative responses were measured as [3H] thymidine incorporation by an automatic beta counter. Tests were performed in triplicates, and results were expressed as the mean cpm.

2.2.4.3 Flow cytometry analysis of human cells

Surface expression was determined using the co-stimulatory molecules CD80 and CD86, the maturation marker CD83, and HLA-DR (BD Pharmingen) (see Table 10). Human DCs were incubated in 1% human AB serum/PBS and incubated with rat anti-CD16/CD32 (BD Pharmingen) to block nonspecific binding. Live cells were gated using forward (FSC) versus side scatter (SSC) dot plots. Samples were measured with the FACS machines LSRII (BD Bioscience, USA). Data obtained were analysed using FACS DiVA software (BD Bioscience, USA) or the software FlowJo Mac v8.84 (Tree Star, Inc., USA).

2.2.5 Statistical analysis

Statistical analyses were performed using the GraphPad software Version 5.03 and are displayed in the results part inside the figure legends. For multiple group comparisons, one-way ANOVA or two-way ANOVA were applied. For comparisons of independent groups, the Student's *t* test or the Mann-Whitney test were performed. For comparisons of matched groups, paired Student's *t* test or Wilcoxon matched test were performed. In figures, n.s. indicates not significant; *** indicates $p < 0.001$; ** indicates $p < 0.01$; and * indicates $p < 0.05$.

3. CHAPTER: RESULTS

3.1 Adjuvanticity of cyclic di-nucleotides

To perform a comparative analysis of the adjuvant properties of cyclic di-nucleotides, mice were vaccinated by mucosal route with the model antigens β -Gal or OVA co-administered with either c-di-AMP, c-di-GMP or c-di-IMP. First, characterization studies were carried out to assess the degree of purity and biological activity of the candidate adjuvants.

3.1.1 Analysis of the purity of the cyclic di-nucleotides with the HEK-Blue™ LPS

Detection assay

The HEK-Blue™ LPS Detection assay is based on the ability of TLR4 to recognize structurally different LPS from Gram-negative bacteria and in particular the lipid A portion, their toxic moiety. Thus, to rule out LPS contamination, which can in term affect both *in vitro* and *in vivo* readouts, the synthesized compounds c-di-AMP, c-di-GMP and c-di-IMP were tested using the HEK-BLUE™ LPS detection kit, according to the manufacturer's instructions. When tested at concentrations up to 10 μ g/ml, the different batches of cyclic di-nucleotides showed no LPS contamination (detection limit, 0.3 ng/ml), as seen in Figure 5.

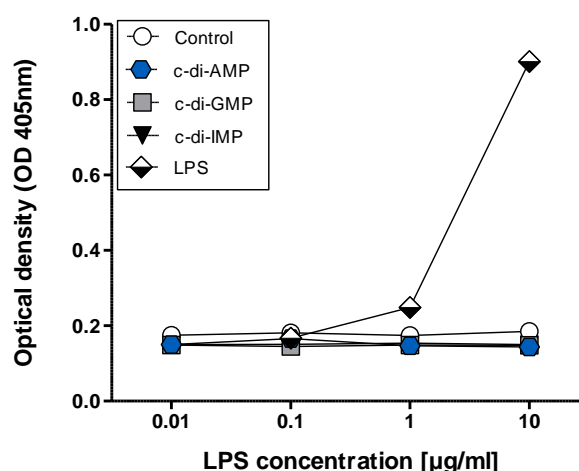


Figure 5: HEK-Blue-based LPS detection.

LPS contamination of the batches from the synthesized compounds was tested by the HEK-BLUE LPS detection kit. The cyclic di-nucleotides showed no LPS contamination.

3.1.2 Analysis of biological activity of the cyclic di-nucleotides - NO-release assay

For the analysis of the potential of cyclic di-nucleotides to induce nitric oxide (NO)-release in macrophages, the cells present in peritoneal exudates from C3H/HeJ LPS non-responder mice were simultaneously stimulated with IFN- γ (62.5 U/ml) and serial dilutions of c-di-AMP, c-di-GMP and c-di-IMP. After a 45 h incubation period, the nitrate is reduced by the nitrite reductase, and the presence of NO is determined as the sum of nitrite and nitrate using the Griss reagent.

The obtained results showed that only c-di-IMP and c-di-AMP induce efficient NO release in pre-activated macrophages (Figure 6). The minimal concentration required for macrophage stimulation by cyclic di-AMP was 60 ng/ml, whereas much higher concentrations of c-di-IMP were needed to induce weak release of NO. No NO release was observed under these experimental conditions when cells were treated with c-di-GMP.

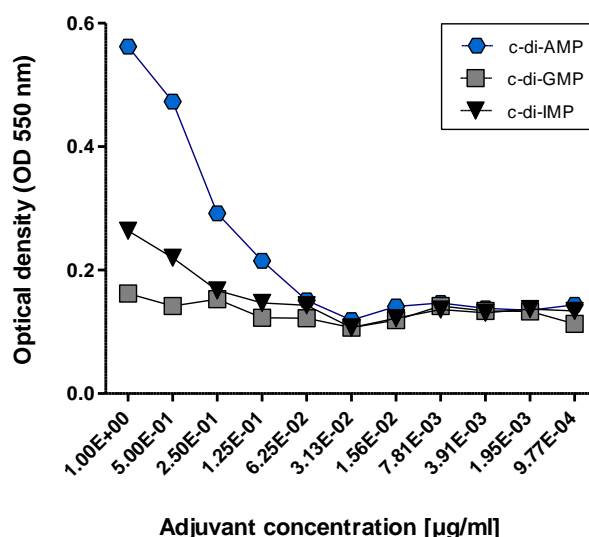


Figure 6: Macrophage activation by c-di-AMP, c-di-GMP and c-di-IMP.

Cells obtained from peritoneal exudates from C3H/HeJ LPS non-responder mice were simultaneously stimulated with IFN- γ (to activate the macrophages) and serial dilutions of the candidate adjuvants c-di-GMP, c-di-AMP or c-di-IMP. Results were expressed as the arithmetic means of the optical densities (OD 550 nm) of triplicates.

3.1.3 The c-di-nucleotides induce strong humoral immune responses after *i.n.*

immunization

To analyse the capacity of the cyclic di-nucleotides to act as a mucosal adjuvant *in vivo*, BALB/c mice were immunized with the model antigen β -Gal (15 μ g/dose) alone or co-administered with c-di-AMP, c-di-GMP or c-di-IMP by the *i.n.* route. The B subunit of the cholera toxin (CTB), which is a well-known adjuvant, was used as a golden standard. The

humoral immune responses stimulated after vaccination were characterized by determining the systemic production of antigen-specific IgG in sera and local IgA, IgM and the secretory component (SC; IgR) in nasal, vaginal and lung lavages by ELISA. As shown in Figure 7, a significant increment in β -Gal-specific IgG-titres was observed in sera from animals receiving the c-di-nucleotides, in comparison to animals vaccinated with the antigen alone or co-administered with CTB.

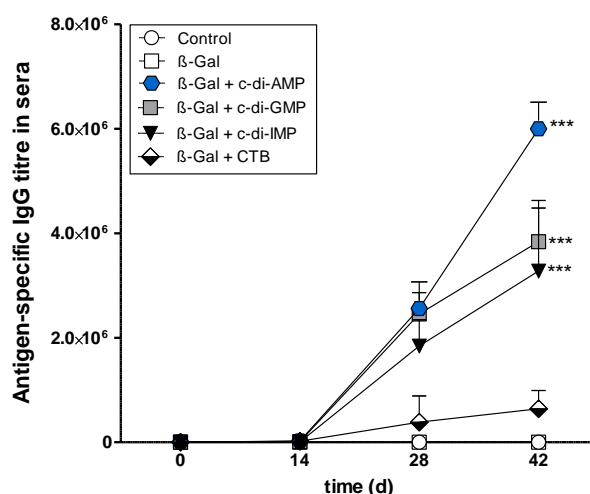


Figure 7: Systemic humoral immune responses stimulated in BALB/c mice vaccinated using c-di-AMP, c-di-GMP and c-di-IMP as mucosal adjuvants.

Kinetic analysis of anti- β -Gal IgG responses in sera from BALB/c mice ($n=5$) immunized on day 0, 14 and 28 with PBS (control), β -Gal alone (15 μ g/dose) or β -Gal co-administered with 5 μ g/dose of c-di-AMP, c-di-GMP, c-di-IMP or CTB by the intranasal route. Differences were statistically significant at $p<0.001$ with respect to BALB/c mice receiving antigen alone (***). One representative out of four independent experiments is shown. The results are expressed as end point titres. The S.E.M. is indicated by vertical lines.

Then, the stimulation of mucosal immune responses by the c-di-nucleotides was evaluated by measuring antigen-specific IgA, IgM and SC titres in nasal, lung and vaginal lavages from vaccinated animals (Figure 8). The i.n. immunization with β -Gal co-administered with c-di-nucleotides induced strong antigen-specific IgA responses in all the analysed mucosal territories. In contrast, antigen-specific IgA was not detected in lavage samples from mice receiving β -Gal alone ($p<0.001$). To rule out the potential contribution of transudating antigen-specific IgA and IgM, the results were further confirmed by an ELISA which specifically detects the polymeric immunoglobulin receptor (i.e. SC). There was a significant increment of antigen-specific Ig with SC in lavage samples from mice vaccinated with the formulations containing c-di-AMP, c-di-GMP or c-di-IMP (Figure 8 A-C).

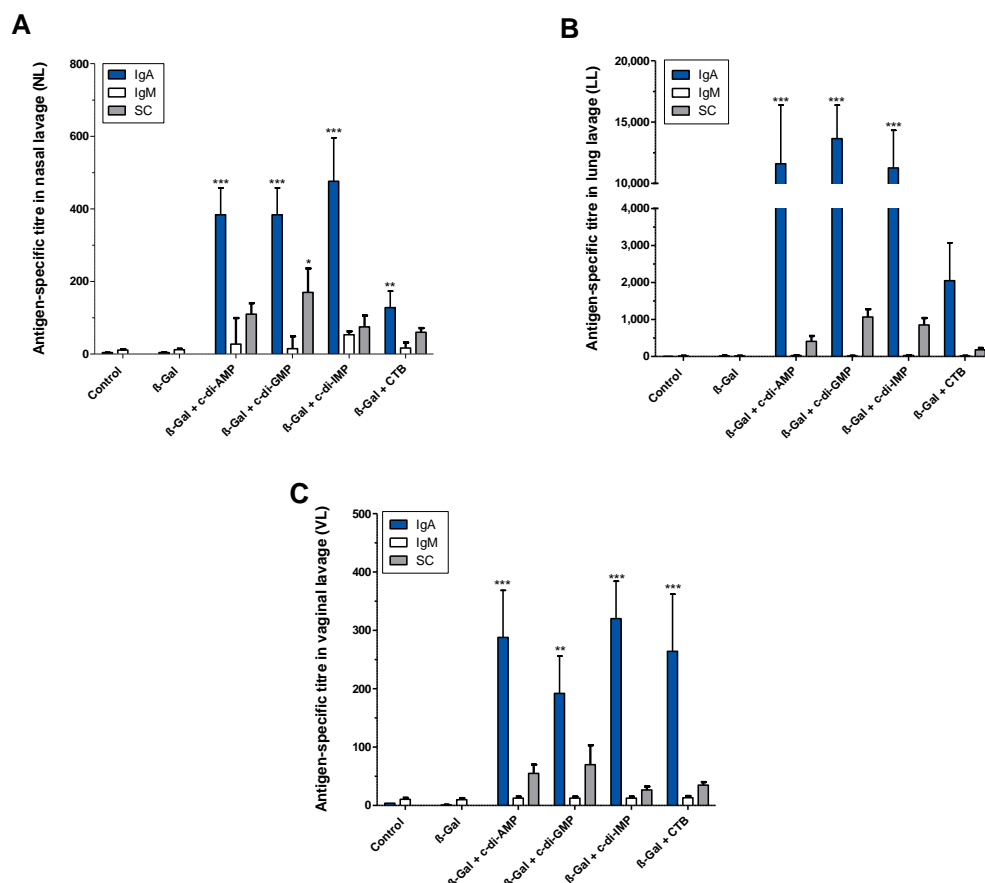


Figure 8: Humoral immune responses stimulated in BALB/c mice vaccinated using c-di-nucleotides as mucosal adjuvant.

Analysis of the antigen-specific IgA, IgM and secretory Ig (sIg) expression **A**) in nasal (NL), **B**) lung (LL) and **C**) vaginal lavages (VL) of immunized mice. Fourteen days after the last immunization lavage samples of vaccinated mice were analysed by ELISA. Results are expressed as end point titres. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***), $p < 0.01$ (**) or $p < 0.05$ (*) with respect to BALB/c mice receiving antigen alone. One representative out of three independent experiments is shown.

3.1.4 Induction of cellular immune responses after *i.n.* immunization

Lymphoproliferative assays were carried out to evaluate the capacity of c-di-nucleotides to promote cellular immune responses. To this end, splenocytes isolated 42 days after the first immunization were re-stimulated *in vitro* with the β -Gal protein. The strongest proliferative responses were observed in cells from mice receiving β -Gal with c-di-AMP followed by those from mice vaccinated using c-di-IMP, CTB or c-di-GMP as adjuvants (Figure 9). In contrast, no responses were observed when splenocytes from mice immunized with the β -Gal protein alone were tested.

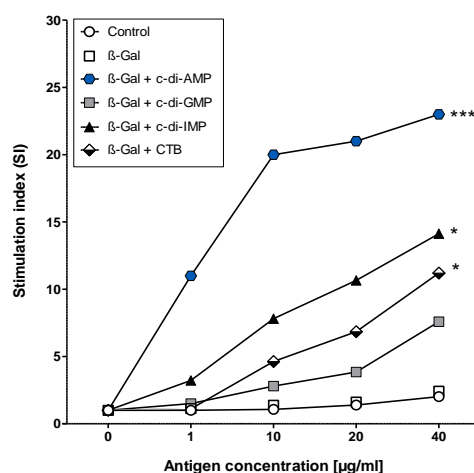


Figure 9: Evaluation of the cellular responses stimulated in mice vaccinated using c-di-nucleotides as mucosal adjuvant.

Spleen cells from vaccinated animals were re-stimulated with different concentrations of β -Gal for 96 h. Cellular proliferation was then assessed by determination of the [3 H]-thymidine incorporated into the DNA of replicating cells. Results are averages of quadruplicates and they are expressed as stimulation indexes (SI). The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***) or $p < 0.05$ (*) with respect to values from cells of BALB/c mice receiving antigen alone. One representative out of three independent experiments is shown.

3.1.5 Stimulation of antigen-specific CD8⁺ T cells after vaccination with c-di-nucleotides as adjuvants

Depending on the pathogen (e.g. viruses), the stimulation of a vigorous cytotoxic T cell response after vaccination could be essential in order to induce efficient protective immunity. Thus, the potential of c-di-nucleotides to elicit cytolytic activity of T cells was analysed. To evaluate whether c-di-AMP, c-di-GMP or c-di-IMP are able to induce antigen-specific cytolytic T cells, C57BL/6 mice were immunized three times with 30 μ g OVA alone or co-administrated with 5 μ g of the c-di-nucleotides by the i.n. route. Four weeks after the last immunization, OVA-peptide loaded CFSE⁺ spleen cells obtained from naïve animals were intravenously injected to the vaccinated mice and the capacity of CTL stimulated by vaccination to lyse them *in vivo* was further evaluated by flow cytometry. The obtained results showed that OVA-specific cytolytic T cells were able to lyse antigen-specific target cells in animals immunized with OVA co-administered with c-di-AMP, c-di-GMP or c-di-IMP. Approximately 64%, 28% and 65% of the peptide loaded cells were lysed *in vivo*, respectively, whereas in mice vaccinated with the antigen alone only a weak (11%) OVA-specific lysis was observed (Figure 10).

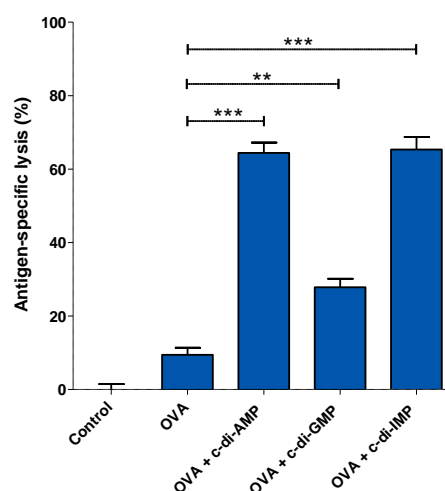


Figure 10: OVA-specific *in vivo* CTL activity in vaccinated mice.

Assessment of the effect of co-administration of c-di-nucleotides on CTL responses as measured by the VITAL assay (*in vivo* CTL). Four weeks after the last boost, the lysis of CFSE⁺, OVA-peptide loaded splenocytes was monitored relative to unloaded splenocytes in immunized mice (n=4). The percentage of specific lysis for each group of four animals is shown. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***) or $p < 0.01$ (**) with respect to BALB/c mice receiving antigen alone. One representative out of three independent experiments is shown.

Furthermore, the adjuvanticity effect of the cyclic di-nucleotides was proven also with the antigen OVA. Therefore, the C57BL/6 mice were immunized as described above and the systemic production of OVA-specific IgG in sera and local IgA in nasal, vaginal and lung lavages were determined by ELISA (Figure 11A-B).

As shown in Figure 11A, a significant increment in OVA-specific IgG-titres was observed in sera from animals receiving the c-di-nucleotides, in comparison to animals vaccinated with the antigen alone.

Moreover, the stimulation of mucosal immune responses by the c-di-nucleotides was evaluated by measuring antigen-specific IgA titres in nasal, lung and vaginal lavages from vaccinated animals (Figure 11B). The i.n. immunization with OVA co-administered with c-di-nucleotides induced strong antigen-specific IgA responses mainly in the nasal cavity. However, an increment of OVA-specific IgA titre in the lung lavage (5-fold) and vaginal lavage (3-fold) samples of animals immunized with OVA+ c-di-AMP could be also observed, in contrast to mice vaccinated with the antigen alone.

Then, proliferative responses were observed in splenocytes derived from OVA vaccinated C57BL/6 mice. Therefore, splenocytes isolated 42 days after the first immunization were re-stimulated *in vitro* with the OVA protein and the proliferation capacity was measured as [³H]-thymidine incorporation. As shown in Figure 11C the strongest proliferative responses were observed in cells from mice receiving OVA with c-di-AMP followed by those from mice vaccinated using c-di-GMP as adjuvants. In contrast, only a

weak response was observed in splenocytes from mice immunized with c-di-IMP as adjuvant and no response when OVA alone were tested.

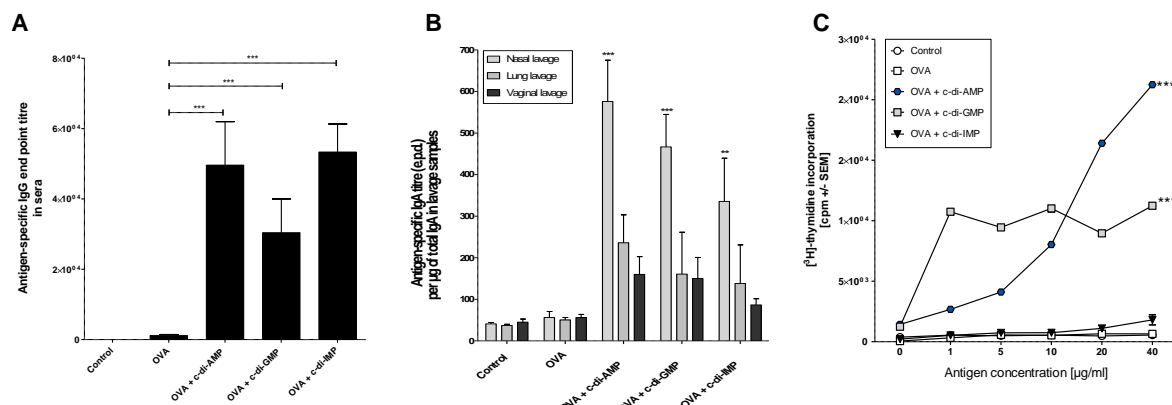


Figure 11: Evaluation of OVA-specific immune responses stimulated in C57BL/6 mice vaccinated using c-di-AMP as mucosal adjuvant.

A) Analysis of OVA-specific serum IgG titres in mice ($n=5$) immunized by the intranasal route three times (day 0, 14 and 28) with PBS, OVA alone (30 μ g per dose) or OVA co-administered with c-di-AMP, c-di-GMP or c-di-IMP (5 μ g) by ELISA. Results are expressed as end point titres. Differences were statistically significant at $p<0.001$ (***) or $p<0.01$ (**) with respect to values in C57BL/6 mice receiving antigen alone. The S.E.M. is indicated by vertical lines. One representative experiment out of three is shown. **B)** Analysis of antigen-specific IgA in nasal, lung and vaginal lavages. Results are expressed as OVA-specific IgA titres per μ g of total IgA. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p<0.05$ (*) with respect to values in C57BL/6 mice receiving antigen alone. **C)** Proliferative responses stimulated in mice immunized using c-di-AMP, c-di-GMP or c-di-IMP as adjuvants. Spleen cells from vaccinated animals were re-stimulated with different concentrations of OVA for 96 h. Cellular proliferation was then assessed by determination of the [³H]-thymidine incorporated into the DNA of replicating cells. Results are averages of quadruplicates and they are expressed as counts per minute (cpm). Differences were statistically significant at $p<0.001$ (***) with respect to values from cells of C57BL/6 mice receiving antigen alone. One representative experiment out of three is shown.

3.1.6 Induction of a balanced Th1/Th2/Th17 T helper immune response after vaccination with c-di-nucleotides as adjuvants

To assess the effect on T helper responses resulting from using c-di-nucleotides as mucosal adjuvant, the subclasses of β -Gal-specific serum IgG were first analysed (Figure 12). The production of IgG1 indicates a Th2 immune response, whereas IgG2a is stimulated during a Th1 dominated response. The obtained results revealed a balanced IgG1/IgG2a response in animals receiving β -Gal co-administered with c-di-nucleotides, whereas in animals receiving only antigen a weak production of antigen-specific IgG subclasses was shown (Figure 12). In contrast, control mice receiving CTB developed a Th2 biased response, characterized by enhanced expression of the IgG1-subclass. These results suggested the stimulation of a balanced Th1/Th2 immune response in mice vaccinated with

c-di-nucleotides. The highest titres were observed in animals vaccinated using c-di-AMP as adjuvant, followed by those immunized with c-di-GMP and c-di-IMP.

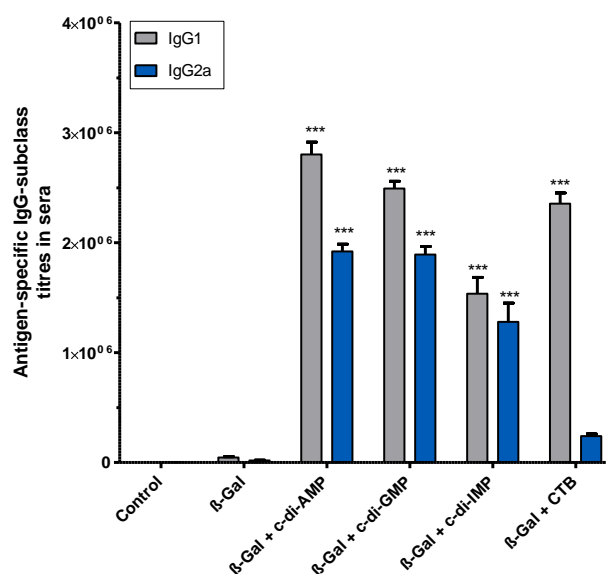


Figure 12: Analysis of antigen-specific IgG isotypes stimulated in vaccinated animals.

Analysis of β -Gal-specific IgG subclasses in sera of immunized mice ($n=5$) by ELISA. Differences were statistically significant at $p<0.001$ with respect to BALB/c mice receiving antigen alone (***). One representative out of four independent experiments is shown. Results are expressed as end point titres. The S.E.M. is indicated by vertical lines.

Then, we analyzed the levels of cytokines secreted by lymphocytes from vaccinated animals. To this end, the presence of β -Gal-specific IFN- γ , IL-2, IL-4 and IL-17 secreting cells was assessed by ELISPOT (Figure 13). Splenocytes from mice immunized with antigen co-administered with c-di-nucleotides predominantly secreted IL-17 in response to β -Gal antigen alone, followed by IL-2, IL-4 and IFN- γ . The higher number of IFN- γ producing cells were observed in cells from animals receiving c-di-GMP as adjuvant. Similar qualitative results were obtained when cells from control mice receiving CTB as adjuvant were tested, but with a much lower absolute number of cytokine producing cells (Figure 13).

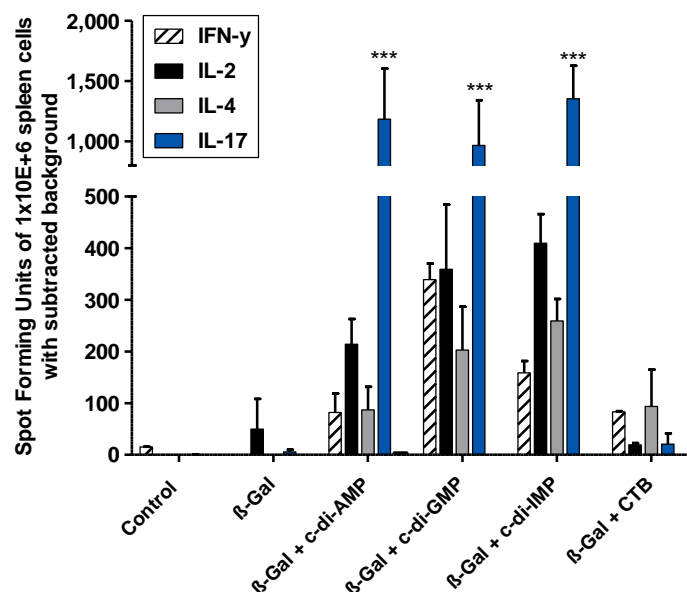


Figure 13: Analysis of Th-specific cytokines in immunized mice.

Spleen cells recovered from vaccinated mice were incubated for 24 or 48 h in the presence of a peptide encompassing the immunodominant Ld-restricted epitope of β -Gal (TPHPARIGL), which is specific for MHC class I presentation (for IFN- γ), or the β -Gal protein (for IL-2, IL-4 and IL-17). Then, the numbers of cytokine producing cells was determined by ELISPOT. Results are presented as spot forming units per 10^6 cells, which were subtracted from the values obtained from non-stimulated cells. One representative experiment out of three is shown. Differences were statistically significant at $p < 0.001$ with respect to values in BALB/c mice receiving antigen alone (***). S.E.M. is indicated by vertical lines.

To further confirm these data, the cytokine secretion pattern of splenocytes from vaccinated animals was determined after *in vitro* re-stimulation. To this end, spleen cells were incubated *in vitro* with different concentrations of β -Gal for 4 days and the concentration of Th1 (IFN- γ , TNF α , IL-2), Th2 (IL-1, IL-4, IL-6, IL-10) and Th17 (IL-17) specific cytokines were analysed (Figure 14 and Figure 15).

In animals vaccinated with β -Gal co-administered with c-di-nucleotides by i.n. route a strong increase of IFN- γ was measured. When cells from animals receiving c-di-AMP as adjuvant were tested, approximately 3-fold higher production of cytokines was observed, as compared to those from mice vaccinated with c-di-GMP or c-di-IMP. Only cells from mice in which β -Gal was co-administered with c-di-IMP showed strong IL-2 production. Concerning Th2 cytokines, an increased secretion of IL-6 and IL-10 was observed in mice immunized with the c-di-nucleotides (Figure 14).

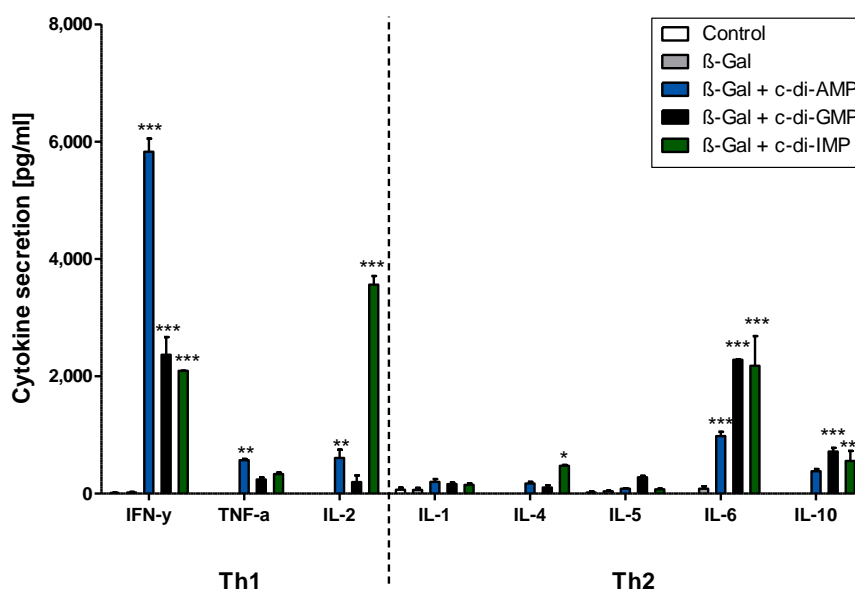


Figure 14: β-Gal-specific cytokine secreting cells in mice immunized with β-Gal co-administered with c-di-nucleotides.

Cells were re-stimulated in quadruplicates with different concentrations of β-Gal for 96 h. The cytokines secreted by spleen cells were then evaluated by a Th1/Th2 Flowcytomix array. Results are expressed in pg/ml. One representative experiment out of three is shown. Differences were statistically significant at $p < 0.01$ (**), $p < 0.01$ (***) or $p < 0.05$ (*) with respect to values of cells from BALB/c mice receiving the antigen alone. S.E.M. is indicated by vertical lines.

The Th17 cytokine IL-17 was strongly up-regulated in the groups of mice immunized with all three c-di-nucleotides. However, mice receiving c-di-AMP as adjuvant showed approximately 2-fold higher levels of IL-17 secretion than those immunized with either c-di-GMP or c-di-IMP (Figure 15).

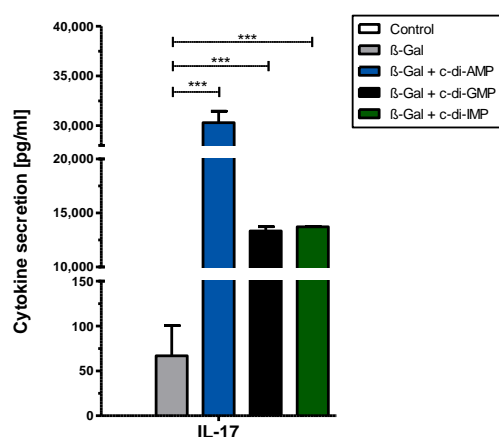


Figure 15: β-Gal-specific IL-17 secretion by cells from mice immunized with β-Gal co-administered with c-di-nucleotides.

Spleen cells were re-stimulated in quadruplicates with different concentrations of β-Gal for 96 h. The cytokines secreted by cells were then evaluated using a Th1/Th2 Flowcytomix array. Results are expressed in pg/ml. One

*representative experiment out of three is shown. Differences were statistically significant at $p < 0.001$ (***) with respect to values from cells of BALB/c mice receiving the antigen alone. S.E.M. is indicated by vertical lines.*

The performed studies demonstrated that immunization with antigen co-administered with c-di-AMP, c-di-GMP or c-di-IMP by the mucosal route resulted in enhanced humoral and cellular immune responses and revealed a balanced Th1/Th2/Th17 immune responses. However, there are some differences in the potency of these small molecules, suggesting that c-di-AMP exerts the most potent adjuvant activity. This makes this compound the most promising for further development. Therefore, the following studies were focussed on the elucidation of the effector functions and underlying mechanisms of action of c-di-AMP.

3.2 *In vitro* studies to assess the effector functions of c-di-AMP on APCs

APCs are cells that convert protein antigens into peptides and present them through MHC complexes on their surface. While all nucleated cells express MHC-class I that allow to present peptides derived antigens, only specialized cells, the APCs (e.g. DCs, MΦs and B cells), have the appropriate machinery for the processing and presentation of extracellular antigens through the MHC-class II molecules. Moreover, APCs are capable of priming and sustaining the expansion of naïve T cells. Further, mature DCs can elicit the activation of other innate immune cells, such as NK cells, by secretion of cytokines (e.g. type I IFNs).

3.2.1 *Activation and maturation of APCs after stimulation with c-di-AMP*

DCs play a central role in bridging innate and adaptive immune responses. Activation can occur via binding of PRR by PAMPs, also defined as danger signals, which are derived from viruses, bacteria, fungi and protozoa. PAMP-mediated stimulation of DCs triggers their differentiation into immunogenic APCs, which are able to prime and sustain the expansion of naïve T cells [128]. Therefore, the effector functions of c-di-AMP on DCs were investigated by evaluating its capacity to trigger differentiation and maturation of bone marrow-derived murine DCs.

The obtained results demonstrated that c-di-AMP was able to promote an efficient activation and maturation of DCs *in vitro*, when used at a final concentration of 1 µg/ml. The analysis by FACS showed an enhanced expression of MHC class II, co-stimulatory (CD80, CD86), and activation (CD40) molecules (Figure 16).

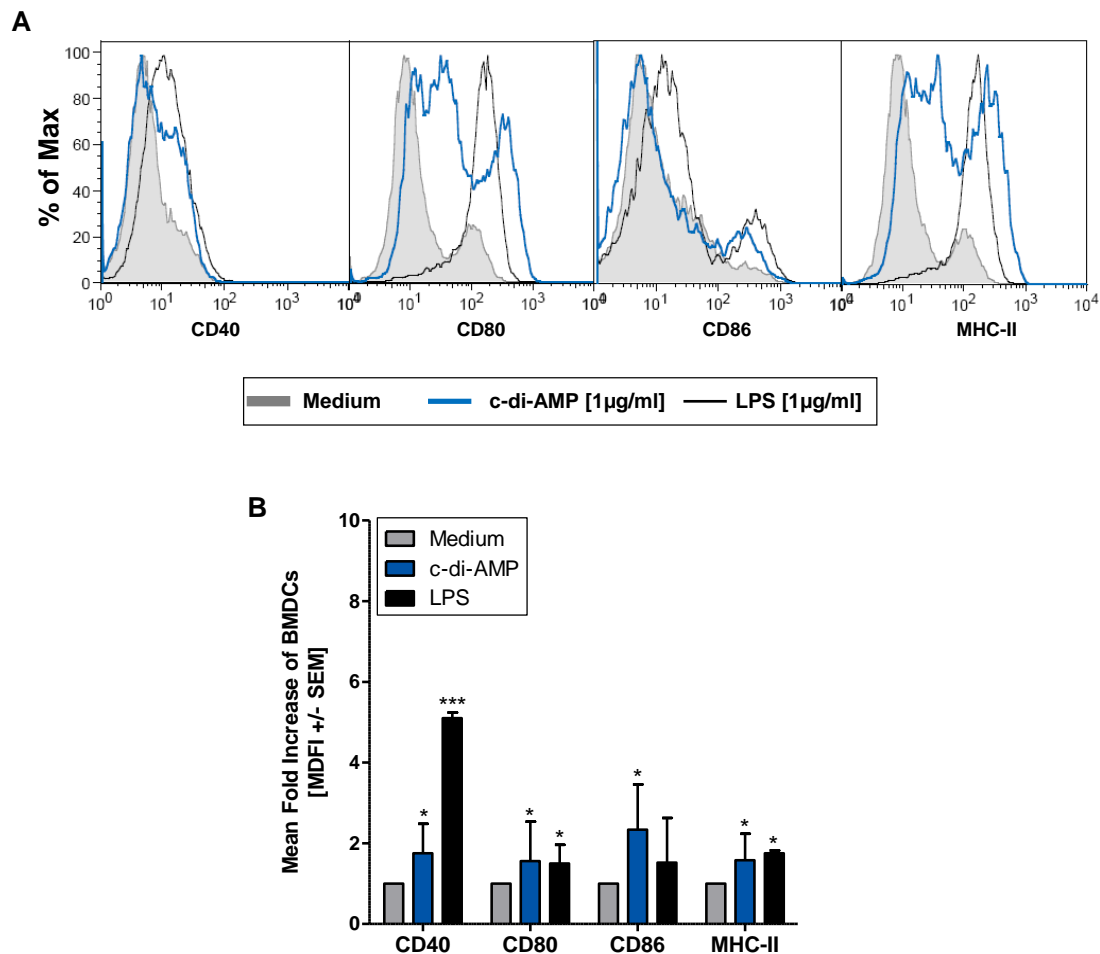


Figure 16: Flow cytometric analysis of DCs after stimulation with c-di-AMP.

A) BM-derived murine DCs were co-incubated with c-di-AMP (1 µg/ml) (blue line) and LPS (black line) for 24 h and analysed by flow cytometry. Cells incubated in medium alone (grey shaded area), were used as controls. The expression of the surface markers CD40, CD80, CD86, and MHC class II on CD11c⁺-gated cells is shown. **B)** The relative expression of each activation marker was expressed as a fold increase: the MDFI (of CD11c⁺ cells) resulting from staining for CD40, CD80, CD86, or MHC class II of stimulated DCs was divided by the MDFI due to staining for the same markers of non-stimulated CD11c⁺ DCs (negative controls). Data represents the mean with the SEM of 4 experiments. Differences were statistically significant at $p < 0.001$ (***) or $p < 0.05$ (*) with respect to non-stimulated DCs.

In order to analyse the capacity of c-di-AMP to stimulate the activation and maturation of murine MΦs, immature bone-marrow derived macrophages (BMMΦ) were stimulated *in vitro* with c-di-AMP for 24 h. As shown in Figure 17, pre-treatment with 1 µg/ml of c-di-AMP for 24 h resulted in an increased expression of CD40, CD86 and MHC-II, in comparison to negative controls and LPS stimulated positive control cells (the analysis was performed gating on CD11b⁺ vital cells).

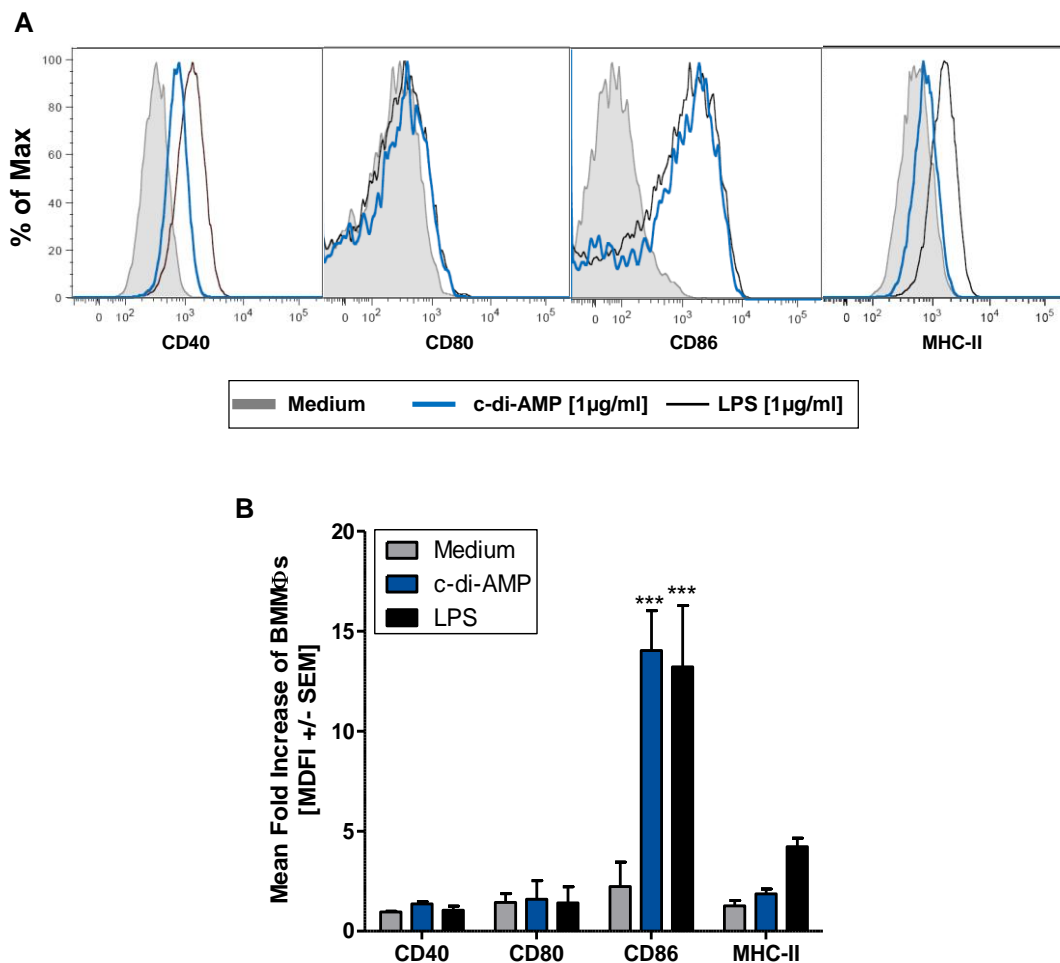


Figure 17: Flow cytometric analysis of BM-derived MΦ after stimulation with c-di-AMP.

A) MΦs were incubated in medium supplemented with c-di-AMP (1 μg/ml) (blue line) or LPS (black line) for 24 h and then analysed by flow cytometry, control cells were culture in medium alone (grey shaded area). The expression of the surface markers CD40, CD80, CD86 and MHC class II on CD11b⁺-gated cells is shown. **B)** The relative expression of each activation marker was expressed as a fold increase: the MDFI (of CD11b⁺ cells) after staining for CD40, CD80, CD86 or MHC class II (for the stimulated DCs) was divided by the MDFI for the same activation markers of non-stimulated CD11b⁺ DCs (negative controls). Data represents the mean of 3 experiments. The SEM is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***) with respect to non-stimulated MΦs.

To further investigate the stimulatory capacities of c-di-AMP on APCs, splenic cells were first sorted for CD19⁺CD21⁺CD23⁺ follicular (FO) B cells, which constitute the majority of B cells found in secondary lymphoid tissue. Then, the sorted B cells were stimulated *in vitro* with c-di-AMP for 5 days. Their proliferation capacity was measured as [³H]-thymidine incorporation (Figure 18A), and the expression of activation markers (Figure 18B) were determined. The treatment of sorted B cells with 1 μg/ml of c-di-AMP resulted in a marginal proliferation. Furthermore, the analysis by FACS showed no increased expression of CD40,

CD80, CD86 and MHC-II markers, in comparison to MALP-2 or LPS stimulated positive control cells.

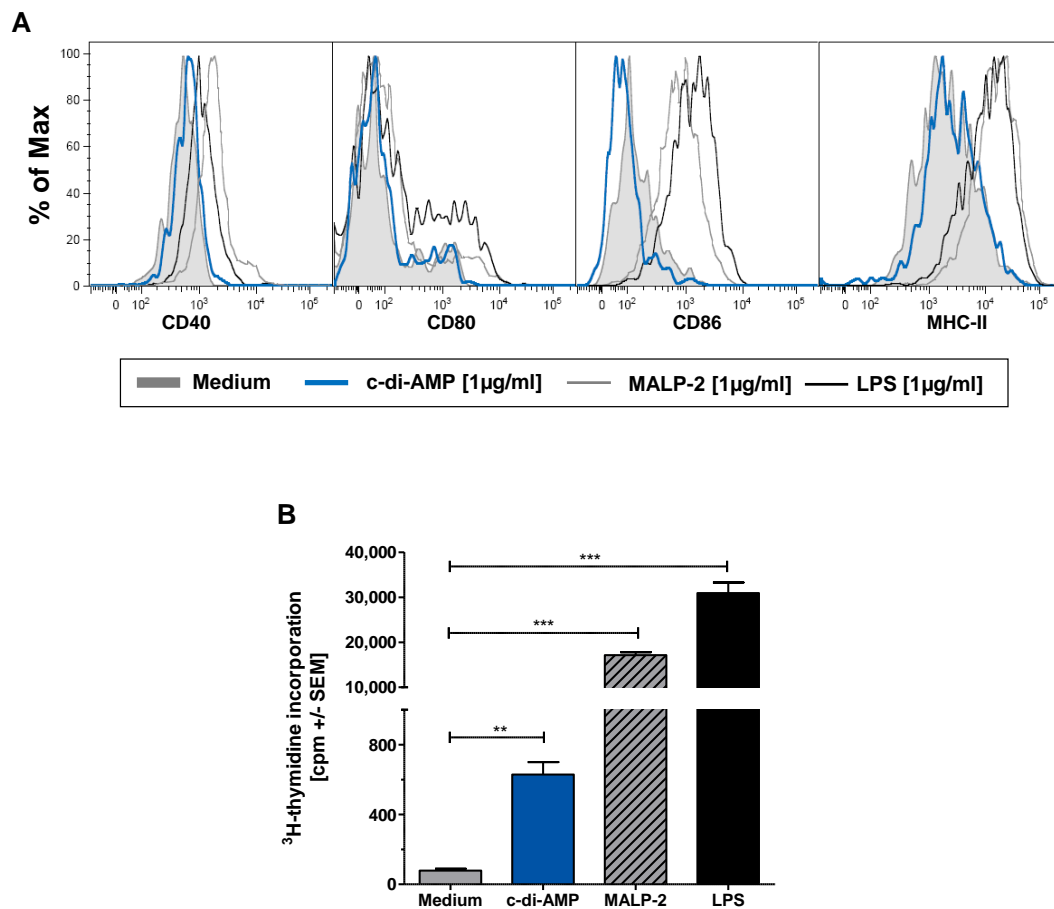


Figure 18: Flow cytometric analysis of B cells after stimulation with c-di-AMP.

A) B cells were incubated in medium alone (grey shaded area) or supplemented with either c-di-AMP (1 µg/ml; blue line), LPS (1 µg/ml; black line) or MALP-2 (1 µg/ml; gray line) for 24 h and then analysed by flow cytometry. The expression of the surface markers CD40, CD80, CD86, and MHC class II on CD19⁺-gated cells is shown. **B)** B cells were incubated in medium alone or supplemented with c-di-AMP, LPS or MALP-2 for 5 days. Then, B cell proliferation was assessed by determination of the [³H]-thymidine incorporated into the DNA of replicating cells. Results are averages of quadruplicates and they are expressed as cpm. Differences were statistically significant at $p < 0.001$ (***) or $p < 0.01$ (**) with respect to non-stimulated B cells. S.E.M. is indicated by vertical lines.

3.2.2 Examination of the T cell proliferation induced by c-di-AMP stimulated DCs

Next, the capacity of DCs loaded with OVA in the presence of c-di-AMP to stimulate proliferation of antigen-specific CD8⁺ and CD4⁺ T cells was evaluated. DCs stimulated with 1 µg/ml c-di-AMP were able to induce a strong proliferation of OVA-specific CD8⁺ from OT-I mice, as shown by the loss of CFSE⁺ stained cells (Figure 19). Furthermore, c-di-AMP pre-activated DCs were also able to stimulate the proliferation of CD4⁺ T cells derived from

DO.11.10 mice. DCs loaded with OVA in the presence of 1 $\mu\text{g/ml}$ LPS were also able to stimulate the proliferation of CD8^+ and CD4^+ T cells. Nevertheless, weaker T cell proliferation was observed when DCs were loaded with the antigen alone.

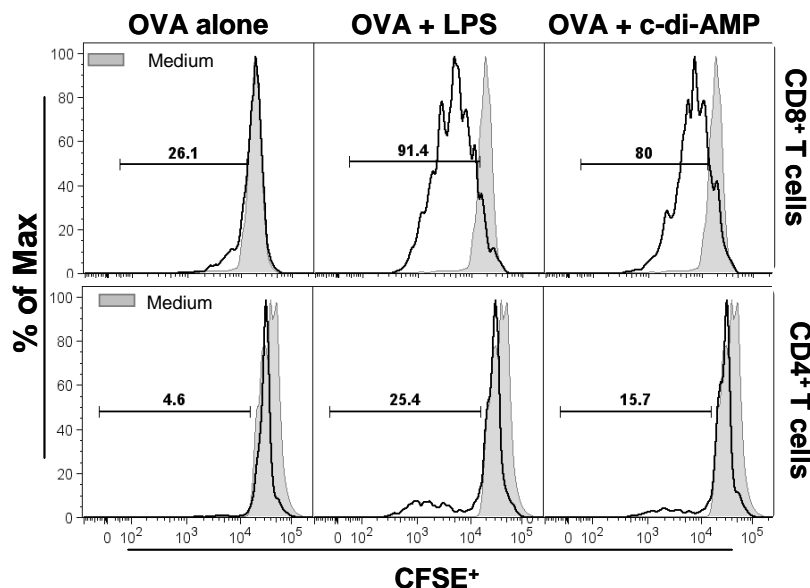


Figure 19: T cell proliferation induced by c-di-AMP stimulated DCs

Bone marrow-derived DCs were incubated for 24 h with LPS-free OVA (10 $\mu\text{g/ml}$) in the presence of c-di-AMP (1 $\mu\text{g/ml}$). Non-stimulated pulsed DCs were used as controls. Then, the loaded DCs were cultured with either CFSE labelled naïve CD8^+ or CD4^+ T cells from OT-I or DO.11.10 mice for 4 days and further analysed by flow cytometry. The results are expressed as histograms and the percentage of the proliferated OT-I and DO.11.10 T cells (black line) with respect to non-stimulated DCs is shown. One experiment out of five independent experiments is shown.

3.2.3 Capability of c-di-AMP to induce a direct CD8^+ and CD4^+ T cell proliferation

Adjuvants can act as non-specific mediators of immune cell function by stimulating or modulating immune cells, but should not have any specific antigenic effect per se. Therefore, we assess the ability of c-di-AMP to stimulate the proliferation of CD4^+ and CD8^+ T cells. To this end, sorted T cells derived from spleens of BALB/c mice were stimulated with or without c-di-AMP (1 $\mu\text{g/ml}$) or ConA (5 $\mu\text{g/ml}$), as positive control. The adjuvant was not able to induce a significant proliferation of specific CD4^+ or CD8^+ , as shown in Figure 20.

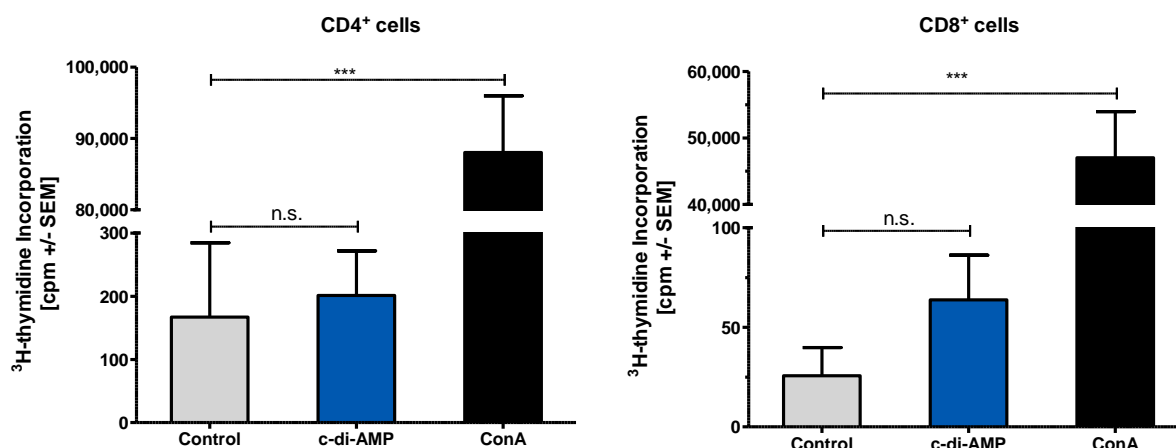


Figure 20: Influence of c-di-AMP on CD4⁺ and CD8⁺ T cells.

Spleen cells from BALB/c mice were sorted for naïve CD4⁺ (CD62L^{high}CD44^{low}CD25⁻) or CD8⁺ (CD62L^{high}CD44^{low}CD25⁻) T cells, which were then left untreated or stimulated for 4 days with either c-di-AMP (1 µg/ml) or ConA (5 µg/ml), as a positive control. Cellular proliferation was then assessed by determination of the [³H]-thymidine incorporated into the DNA of replicating cells. Results are averages of quadruplicates and they are expressed as cpm. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***) or non-significant (n.s.) with respect to non-stimulated T cells.

3.2.4 In depth analysis of effector functions of c-di-AMP on different DCs subsets

The initial studies on the effector functions of c-di-AMP on different cell population suggested that DCs represent the main target for the observed immune modulatory activities. Thus, we next examined its effector functions on specific DC subsets. To this end, BMDCs were pre-incubated with Flt3L to promote their differentiation in a mixed DC population. After 9 days of differentiation the cells were stimulated with c-di-AMP or LPS as positive control for 24 h and stained for the specific subset markers CD11c⁺CD11b⁺ from cDCs, which consist of different subtypes including CD8⁻ myeloid DCs (mDCs) and CD8⁺ lymphoid DCs (IDCs), and the CD11c⁺CD11b⁻B220⁺ pDCs (Figure 21).

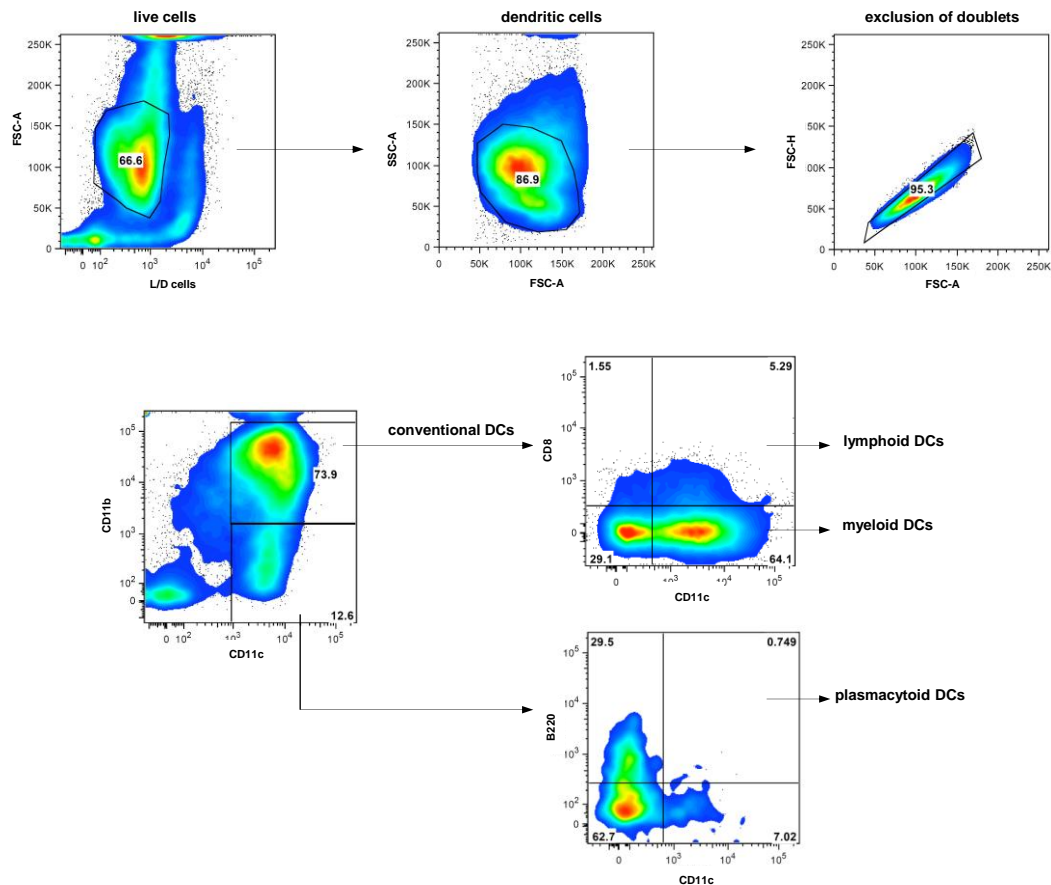


Figure 21: Gating strategy used to identify DC subsets stimulated in the presence or absence of c-di-AMP or LPS.

The percentage of each boxed cell population is indicated. Doublets were excluded by forward scatter area (FSC-A) versus forward scatter height (FSC-H). Dead cells were excluded by their staining with a fluorescent reactive dye.

The effect of c-di-AMP on DC subsets was then investigated in terms of differentiation and maturation of murine BMDC subsets. The c-di-AMP was able to promote an efficient activation and maturation of cDCs *in vitro* when used at a final concentration of 5 $\mu\text{g/ml}$. The analysis by flow cytometry showed a significant expression of the co-stimulatory markers CD80 and CD86 in IDCs, followed by mDCs. Only a weak expression of the CD86 molecule and no up-regulation of the CD80 molecule were observed in pDCs after c-di-AMP stimulation (Figure 23 A-C). LPS was also able to stimulate all DC subsets at a concentration of 1 $\mu\text{g/ml}$.

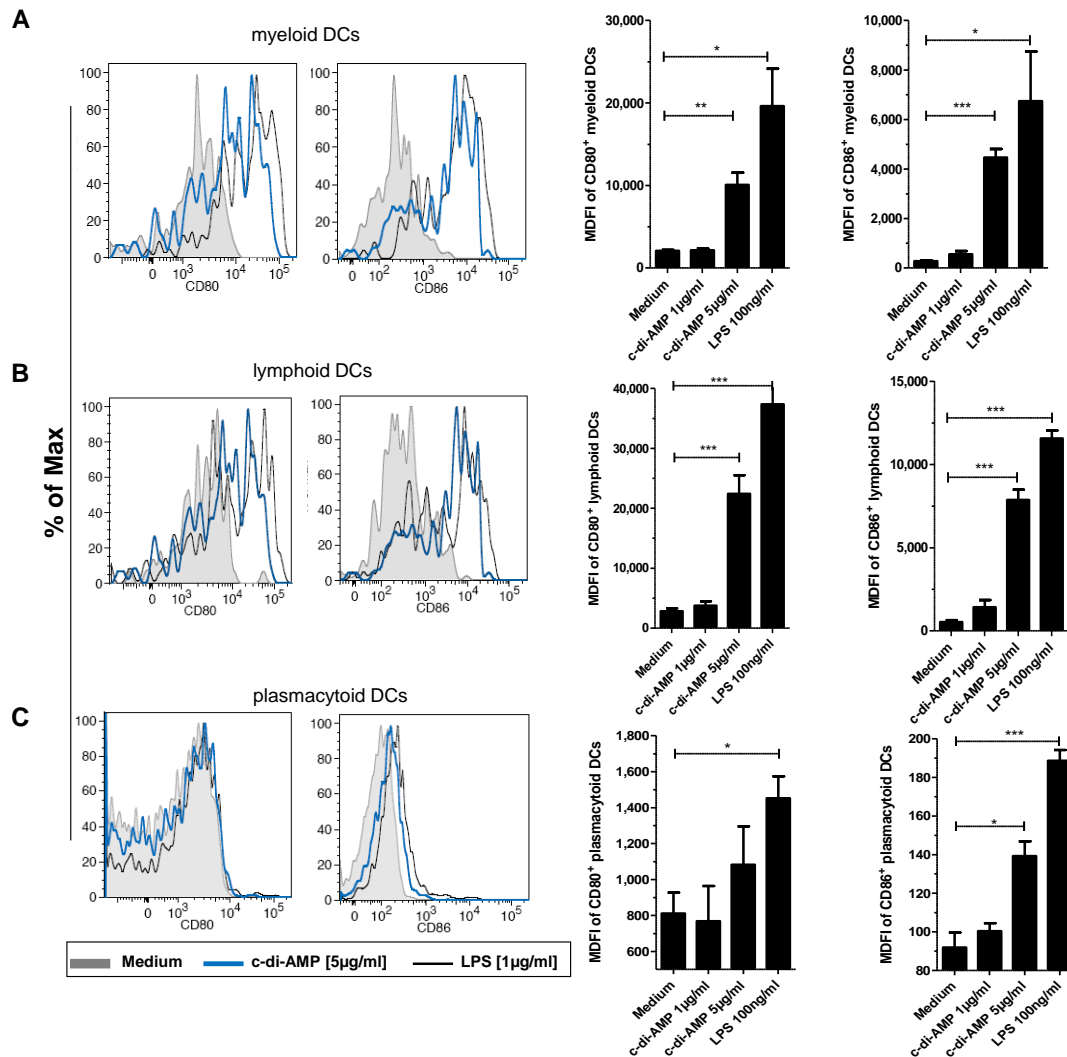


Figure 22: Flow cytometric analysis of DC subsets after stimulation with c-di-AMP in vitro.

BMDCs from BALB/c mice were differentiated by Flt3L for 9 days. Cells were then stimulated with or without c-di-AMP (1 µg/ml and 5 µg/ml) or LPS (1 µg/ml) for 24h. DCs were then stained using a cell viability markers and Abs specific for the DC subsets. The plotted data represent histograms and the average of **A**) CD11c⁺CD11b⁺CD8⁺ MHC-II⁺ mDCs, **B**) CD11c⁺CD11b⁺CD8⁺ MHC-II⁺ IDCs, and **C**) CD11c⁺CD11b⁺B220⁺MHC-II⁺ pDCs expressing the co-stimulatory markers CD80 and CD86. One representative histogram of non-stimulated DCs (grey shaded area), c-di-AMP stimulated DCs (blue line), and/or LPS stimulated DCs (black line). Differences were statistically significant at $p < 0.001$ (***), $p < 0.01$ (**) or $p < 0.05$ (*) with respect to non-stimulated DCs.

3.2.5 Quantification of intracellular cytokine production by DC subsets after c-di-AMP stimulation

The capacity of mature DCs to prime naïve T lymphocytes and to promote their differentiation into different T cell subsets is not only attributed to the up-regulation of surface MHC, co-stimulatory and adhesion molecules, but also on the ability to secrete cytokines

such as IL-1, IL-6, IL-7, IL-12, IL-15, and IL-18. In fact, the secretion of IL-12 is critical for immune responses against many viruses and intracellular bacterial pathogens. The release of IL-12 by DCs increases NK cell- and T cell mediated cytotoxicity and stimulates IFN- γ production and proliferation of NK cells and T cells. Therefore, the pattern of cytokine expression during DC subset stimulation with c-di-AMP or LPS was assessed by intracellular staining, followed by flow cytometric analysis. The DC subsets were defined as described above in the gating strategy (Figure 21).

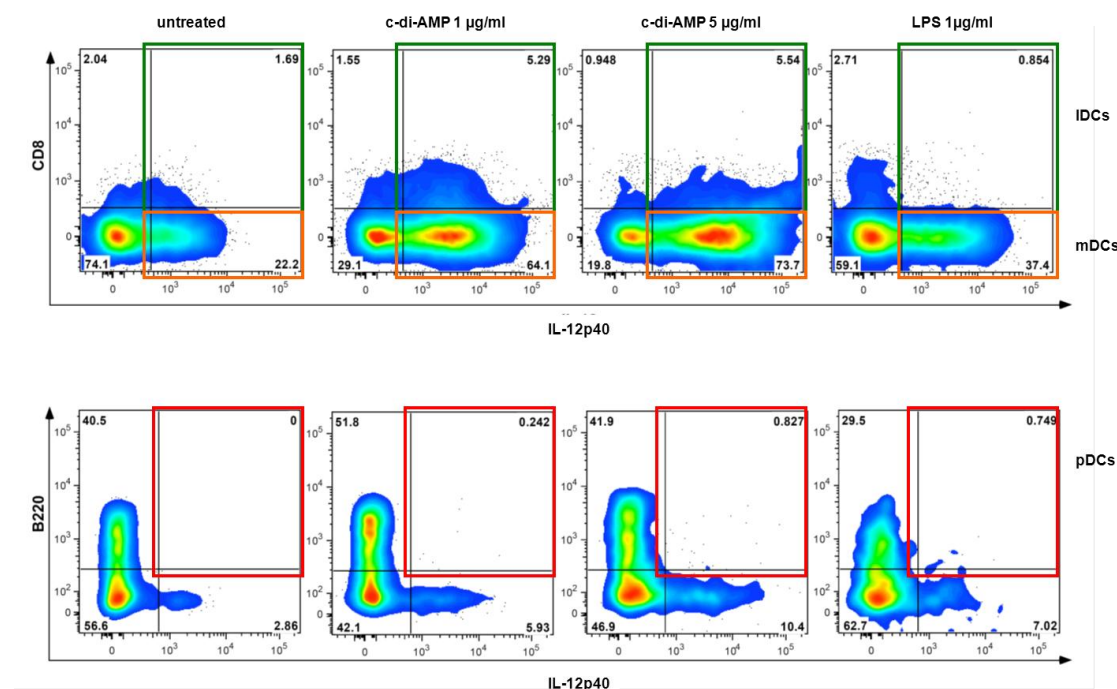


Figure 23: Detection of intracellular cytokine production by DC subsets stimulated with c-di-AMP.

BMDCs from BALB/c mice were differentiated by Flt3L for 9 days. Cells were then stimulated with or without c-di-AMP (1 µg/ml and 5 µg/ml) or LPS (1 µg/ml) for 20 h. DCs were then stained using a cell viability markers and Abs specific for CD11c, CD11b, CD220 and CD8. After permeabilization, intracellular staining was performed using Abs specific for IL-12p40. The plotted data represent the percentage of CD11c⁺CD11b⁺CD8⁺ IDCs (green frame), CD11c⁺CD11b⁺CD8⁻ mDCs (orange frame), and CD11c⁺CD11b⁺B220⁺ pDCs (red frame) producing IL-12p40. The numbers within the quadrants indicate the percentages of cells.

The obtained results showed an increased secretion of IL-12p40 by all three DC subsets after stimulation with 5 µg/ml of c-di-AMP (Figure 23 and Figure 24). However, a strong secretion was observed in the cDC subsets (mDCs and IDCs) even with lower concentration of 1 µg/ml c-di-AMP. In contrast, marginal amount of IL-12p40 are secreted by pDCs after c-di-AMP stimulation. Stimulation with LPS resulted in a lower cytokine production as compared to c-di-AMP, moreover, the LPS stimulated IDCs showed an inhibition of IL-12p40 production (Figure 23 and Figure 24).

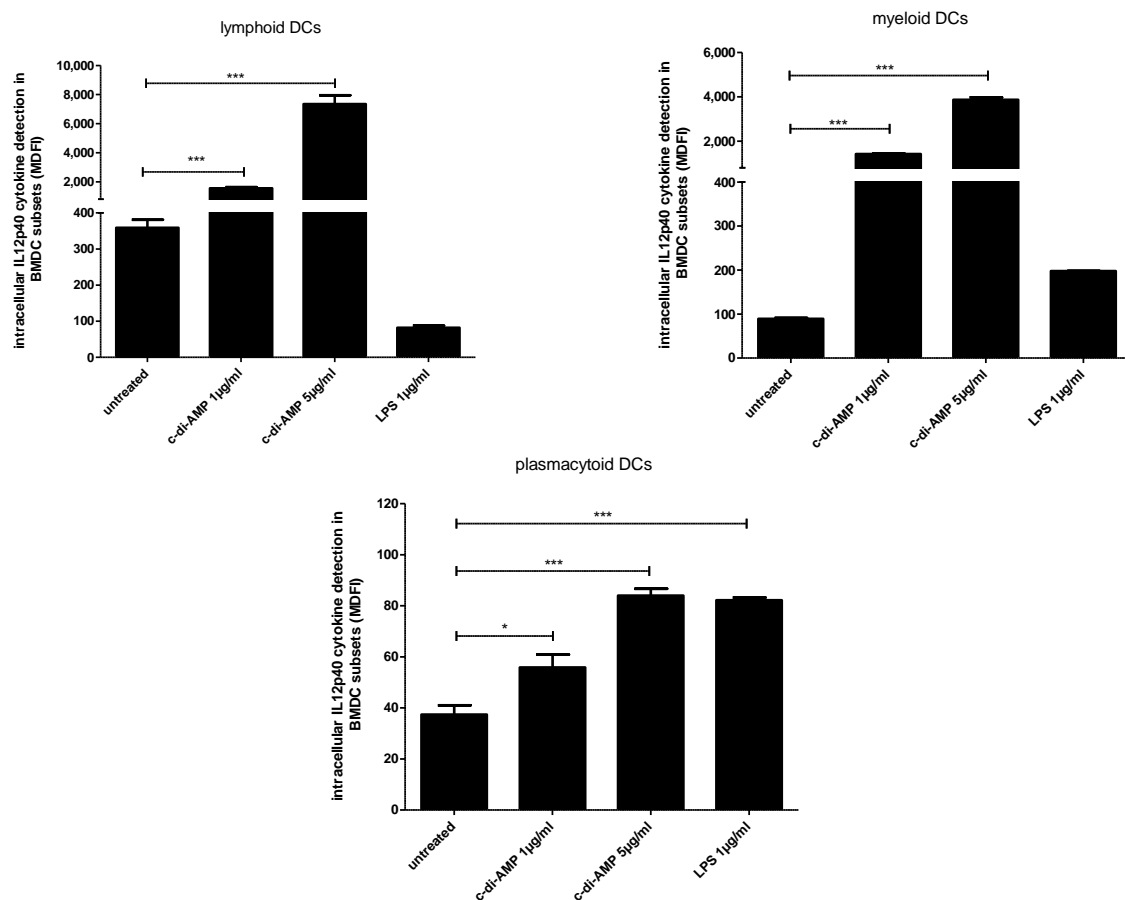


Figure 24: Detection of intracellular IL-12 in BMDC subsets stimulated with c-di-AMP.

BMDs from BALB/c mice were differentiated by Flt3L for 9 days. After DC stimulation with or without c-di-AMP (1 µg/ml and 5 µg/ml) or LPS (1 µg/ml) for 20 h, cells were stained using a cell viability marker and Abs specific for CD11c, CD11b, B220 and CD8. After permeabilization, intracellular staining was performed using Abs specific for IL-12p40. The bars represent the median fluorescence intensity (MDFI) of CD11c⁺CD11b⁺CD8⁺ IDCs, CD11c⁺CD11b⁺CD8⁻ mDCs, and CD11c⁺CD11b⁻B220⁺ pDCs producing IL-12p40. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***) or $p < 0.05$ (*) with respect to non-stimulated DCs.

Next, the intracellular secretion of the IL-10 cytokine was investigated in cDCs. IL-10 can inhibit the release of IL-12, thereby down regulating the Th1 response. The obtained results showed an increased secretion of IL-10 in cDC after stimulation with either 1 µg/ml or 5 µg/ml of c-di-AMP (Figure 25). Treatment on control cells with LPS does not result in any significant increase of IL-10 production in comparison to untreated DCs.

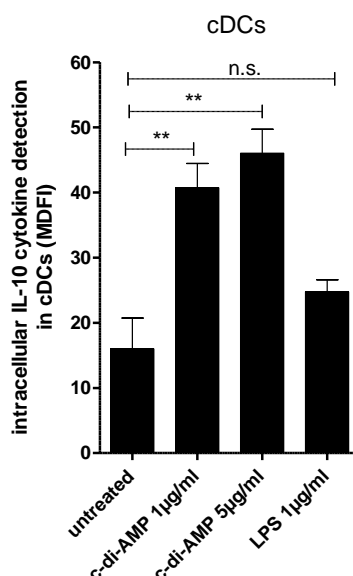


Figure 25: Detection of the intracellular IL-10 in BMDC subsets stimulated with c-di-AMP.

BMDCs from BALB/c mice were differentiated by GM-CSF for 5 days. After DC stimulation with or without c-di-AMP (1 µg/ml and 5 µg/ml) or LPS (1µg/ml) for 20 h, cells were stained using a cell viability marker and Abs specific for CD11 and CD11b. After permeabilization, intracellular staining was performed using Abs specific for IL-10. The bars represent the median fluorescence intensity (MDFI) of CD11c⁺CD11b⁺ cDCs producing the IL-10 cytokine. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.01$ (**) or non-significant (n.s.) with respect to non-stimulated cells.

3.3 Cyclic di-AMP exhibits strong stimulatory activity on human APCs *in vitro*

Since c-di-AMP was showing a strong activation of murine DCs and MΦs it was essential to investigate the ability of c-di-AMP to induce the maturation and activation of human DCs.

3.3.1 Human DC and MΦ activation and maturation after c-di-AMP treatment

The potency of c-di-AMP to activate also human derived DCs and MΦs *in vitro* was investigated. First, human monocyte-derived DCs were prepared from PBMCs and cultured in the presence of GM-CSF for 5 days. Then, immature DCs were treated with different concentrations of c-di-AMP to define the amount of adjuvant needed for the up-regulation of the MHC class II (HLA-DR), co-stimulatory (CD80, CD86) and maturation (CD83) molecules (Figure 26 A-B). The obtained results showed an enhanced expression of MHC-II, CD80/86 and CD83 starting at a concentration of 66 µg/ml of c-di-AMP.

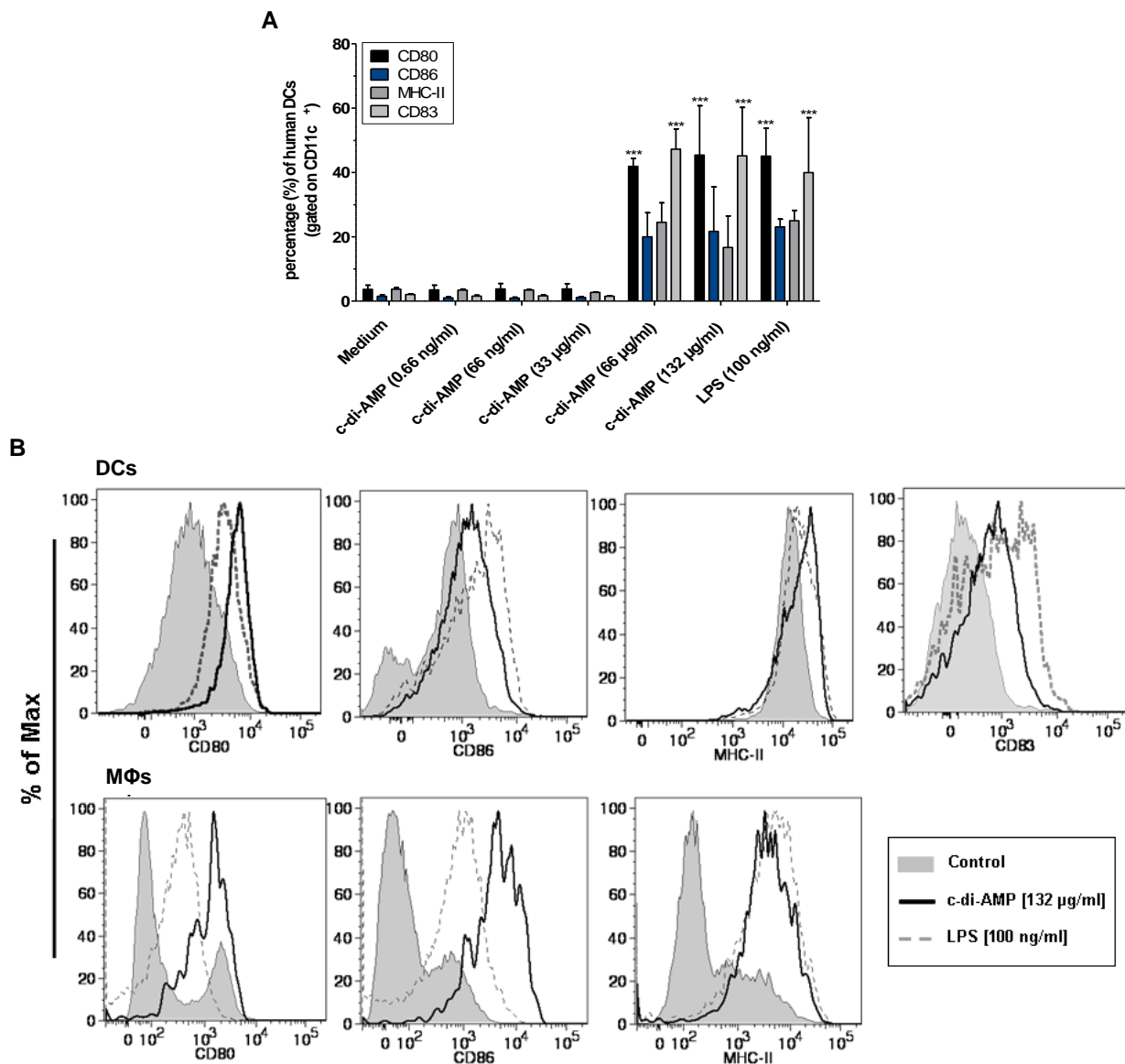


Figure 26: Flow cytometric analysis of human DCs and MΦs after stimulation with c-di-AMP.

PBMC-derived human DCs or MΦs were incubated for 24 h alone or in the presence of LPS, or c-di-AMP. **A)** Dose response of DCs to c-di-AMP stimulation for the CD80, CD86, and MHC-II molecules and maturation marker CD83 was investigated. **B)** PBMC-derived human DCs or MΦs were incubated for 24 h alone (grey shaded) or in the presence of either LPS (100 ng/ml, grey dotted line) or c-di-AMP (132 µg/ml; black line). Cells were then stained with fluorochrome conjugated Abs specific for CD80, CD83 (only for DCs), CD86, and MHC-II (HLA-DR), and further analysed by flow cytometry. Results correspond to an experiment performed with cells from a single donor and they are representative of three independent experiments tests.

Afterwards, PBMC-derived human DCs and MΦs were treated with 132 µg/ml c-di-AMP and 100 µg/ml LPS. As shown in Figure 26B a strong enhanced expression of the examined molecules could be observed in both, DCs and MΦs, after the treatment with c-di-AMP.

3.3.2 Allogeneic T cell proliferation is induced by c-di-AMP stimulated DCs

Allogeneic proliferation is a phenomenon, which was originally discovered in organ transplantation by mediating rejection. It is based on the ability of T cells to recognize non-self MHC complexes leading to an Ag-independent proliferation. Roughly 1-10% of these T cells in an individual respond to stimulation with cells from another unrelated individual [32].

To determine whether the c-di-AMP-treated human DCs have an enhanced ability to stimulate T cells, an allogeneic T cell proliferation assay was performed. Immature DCs, c-di-AMP-treated DCs, and LPS-treated DCs were co-cultured with CFSE-labelled CD4⁺ or CD8⁺ T cells for 6 days, and then analysed by flow cytometry.

The co-culture of c-di-AMP stimulated DCs resulted in a significant proliferation of naïve CD4⁺ T cells (DCs to T cell ratio 1:10) of about 36% (Figure 27). In contrast, un-stimulated DCs induced a proliferation of 25%, whereas LPS matured DCs induced a proliferation of 44%. However, at higher DC:T cell ratios similar proliferation was observed for DCs treated or not with c-di-AMP. In contrast to LPS-treated DCs, no increased proliferation of CD8⁺ T cells was observed when DCs were co-cultured with c-di-AMP.

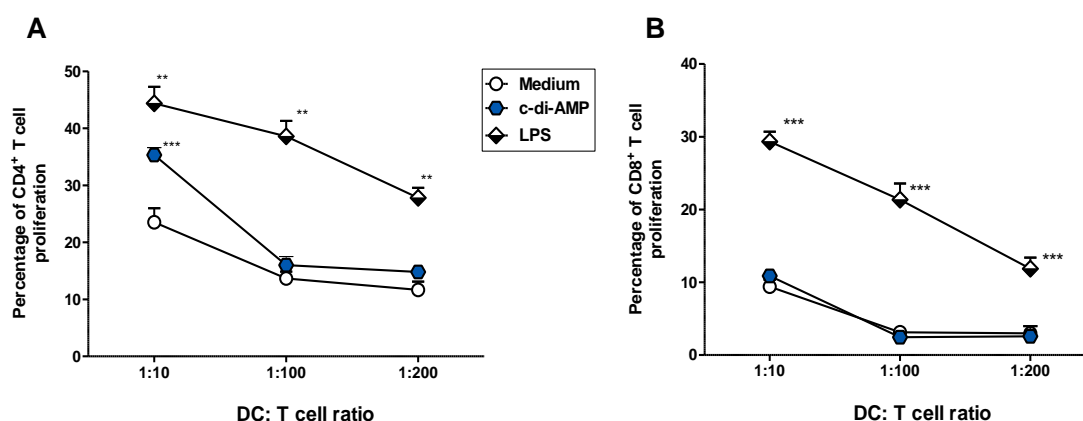


Figure 27: Allogeneic proliferation assay with human DCs and T cells.

Human immature DCs were stimulated with c-di-AMP (132 µg/ml) or LPS (100 ng/ml) or left untreated (medium). After 24 h, DCs were co-cultured with purified CD4⁺ (A) and CD8⁺ (B) T cells at different ratios. On day 4 the cells were labelled with CFSE and analysed by FACS. Differences were statistically significant at $p < 0.001$ (***), or $p < 0.01$ (**) with respect to untreated DCs. S.E.M. is indicated by vertical lines.

3.4 *In vivo* analysis of the immune stimulatory capacities of c-di-AMP

As already shown in the *in vitro* analysis, c-di-AMP was able to stimulate MΦs, DCs and DC subsets very efficiently. Thus, the ability of c-di-AMP to stimulate MΦs, DCs and their subsets *in vivo* was further investigated. To this end, BALB/c mice received c-di-AMP co-administrated with or without β-Gal by i.n. route or MALP-2 as control. After 24 h, the expression of activation and maturation markers by DCs and MΦs from NALT and cLN was analysed *ex vivo* by flow cytometry.

3.4.1 Activation and maturation of DCs and MΦs is induced *in vivo* by c-di-AMP

The c-di-AMP was able to promote an efficient *in vivo* activation and maturation of DCs and MΦs in NALT when used at a concentration of 5 µg/dose (Figure 28). The analysis by FACS showed an enhanced expression of MHC class II, CD80 and CD86 in both DCs and MΦs.

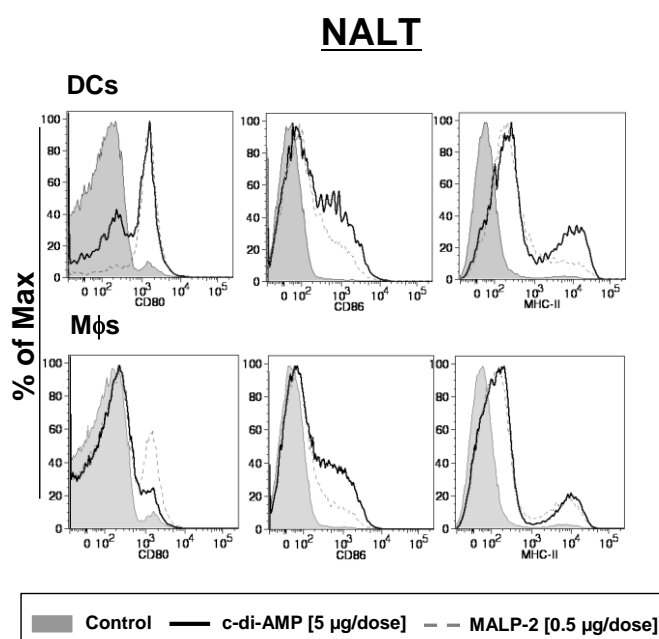


Figure 28: Flow cytometric analysis of DCs and MΦs in NALT after intranasal administration of c-di-AMP.

Balb/c mice were intranasally treated with c-di-AMP (5 µg/dose; black line), MALP-2 (0.5 µg/dose; black dotted line) or PBS (grey shaded area). After 24 h, cells were isolated from the NALT and the expression of the surface markers CD80, CD86 and MHC class II was determined on CD11c⁺-gated cells (DCs) or CD11b⁺-gated cells (MΦ) by flow cytometry. One representative experiment out of three is shown.

Activation and maturation was also observed in DCs from cLN of c-di-AMP-treated mice. In contrast, in MΦs only the expression of CD80 and to less extends MHC class II were clearly up-regulated after treatment with c-di-AMP or MALP-2 (Figure 30).

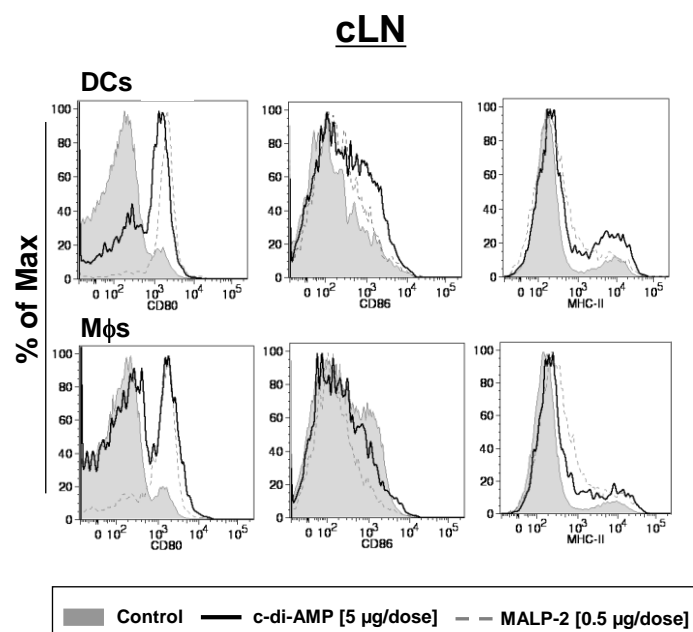


Figure 29: Flow cytometric analysis of DCs and MΦs in cLN after i.n. administration of c-di-AMP.

Balb/c mice were intranasally treated with c-di-AMP (5 µg/dose; black line), MALP-2 (0.5 µg/dose; black dotted line) or PBS (grey shaded area). After 24 h, cells were isolated from the cLN and the expression of the surface markers CD80, CD86 and MHC class II was analysed on CD11c+ (DCs) or CD11b+ (MΦs) gated cells by flow cytometry. One representative experiment out of three is shown.

3.4.2 Activation and maturation of DCs subsets in NALT and cLN after treatment with c-di-AMP

The ability of c-di-AMP to stimulate *in vivo* DCs and their subsets was then examined. BALB/c mice were treated with c-di-AMP alone or co-administered with a model antigen by i.n. route and after 24 h the activation and maturation of DC subsets from NALT and cLN were analysed by flow cytometry. The obtained results showed that c-di-AMP was able to promote a significant increase in the expression of the co-stimulatory molecule CD86 and MHC-II in both cDCs and pDCs from NALT (Figure 30). Despite the observed up-regulation trend for CD80, the differences were not statistically significant. Co-administration of the adjuvant with an antigen does not affect the observed activation pattern.

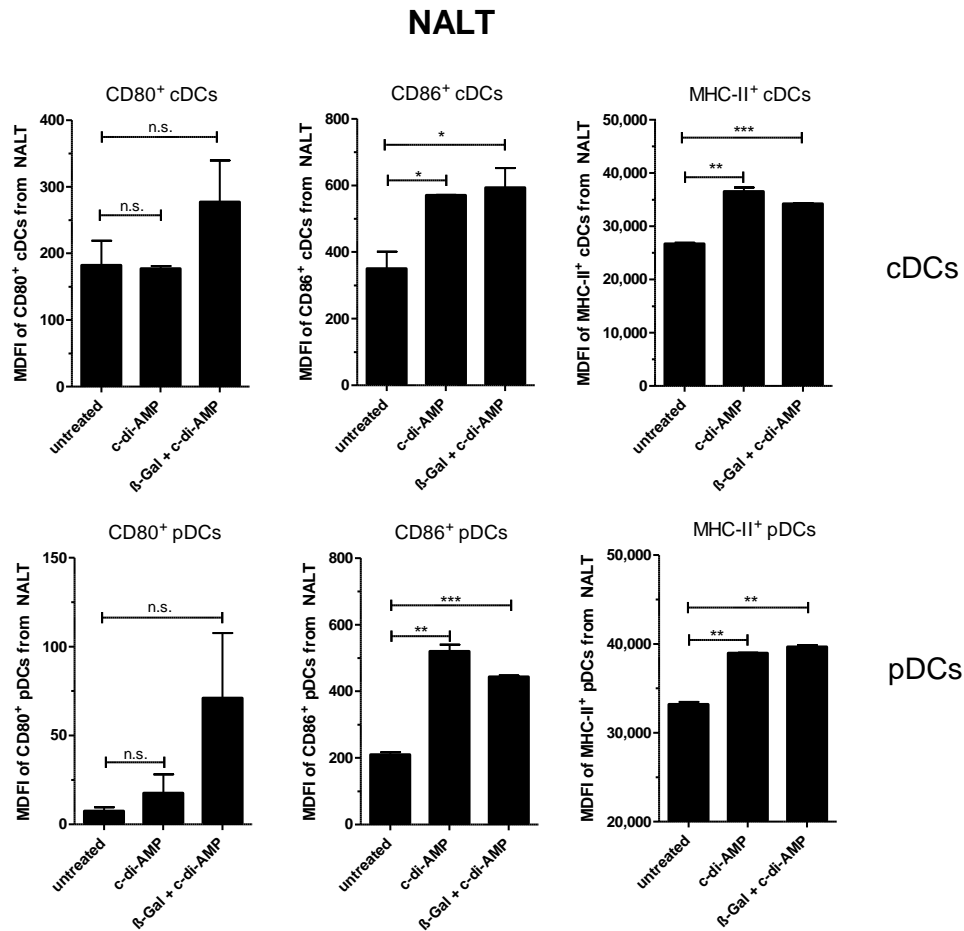


Figure 30: Activation and maturation of DC subsets in NALT after treatment with c-di-AMP.

BALB/c mice were treated with c-di-AMP alone or co-administered with β -Gal by i.n. route. After 24 h, cells isolated from NALT were stained and gated using markers specific for different DC subsets and further analysed for the expression of CD80, CD86 and MHC class II. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*) or non-significant (n.s.) with respect to values from cells of untreated BALB/c mice. One representative out of three independent experiments is shown.

The DCs subset activation in cLN of c-di-AMP-treated mice was quite similar to the results obtained for DCs from NALT. Also here we observed a significant up-regulation of CD86 and MHC-II in both cDCs and pDCs (Figure 30). No significantly up-regulated expression of CD80 was detected after c-di-AMP treatment, and the presence of the antigen has no significant influence on the DC activation pattern.

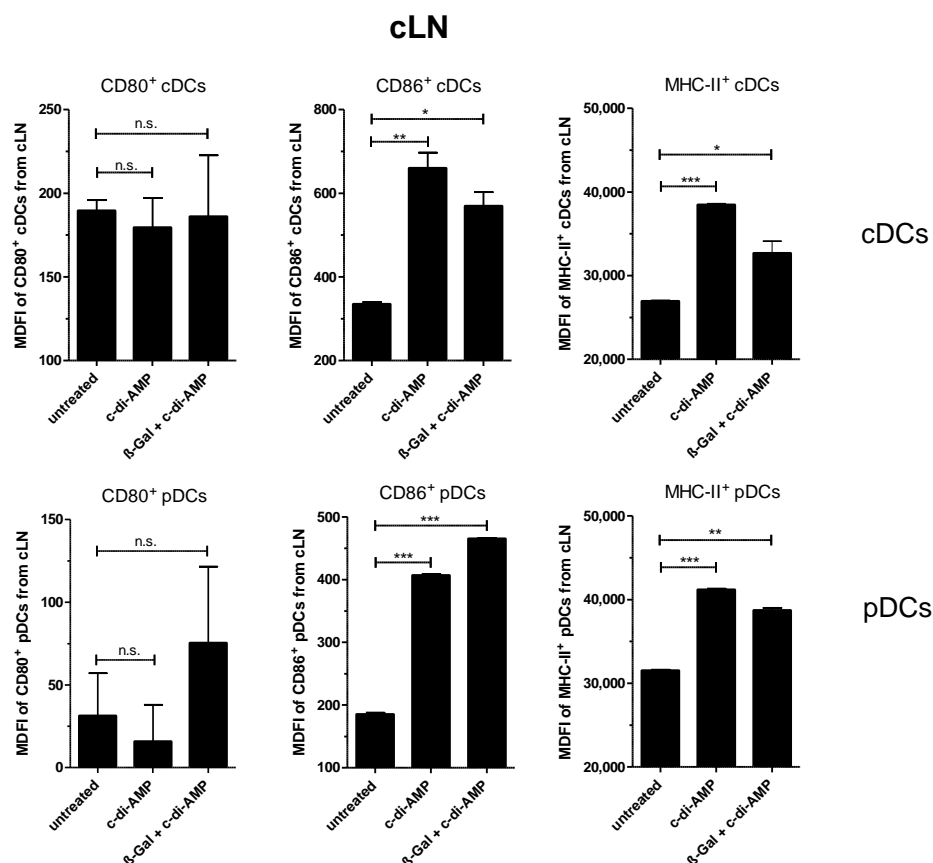


Figure 31: Activation and maturation of DC subsets in cLN after treatment with c-di-AMP.

BALB/c mice were treated with c-di-AMP alone or co-administered with β -Gal by i.n. route. After 24 h cells isolated from cLNs the stained and gated using makers specific for different DC subsets and analysed for the expression of the surface markers CD80, CD86 and MHC class II. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*) or non-significant (n.s.) with respect to cells from untreated BALB/c mice. One representative out of three independent experiments is shown.

3.5 Elucidation of the underlying mechanisms of action of c-di-AMP

It is clear that adjuvants cannot only potentiate immune responses, but can also modulate them. Thus, the understanding their mechanism of action is critical to make optimal use of them, as well as to foresee potential safety issues. Therefore, it is important to identify cellular receptors and elucidate downstream signalling cascades.

3.5.1 Evaluation of putative contribution of cyclic AMP to the adjuvant activity

The c-di-AMP is formed by diadenylate cyclase (DAC) of cyclic AMP (cAMP), which is known to exert different effector functions on eukaryotic cells [112, 129]. For example, transferring the effects of hormones (such as glucagon and adrenaline), and regulating the

passage of Ca^{2+} through ion channels [36, 130]. Moreover, cAMP was shown to be involved in cell proliferation and the regulation of leukocytes function [131]. Thus, it was important to rule out if part of the observed activities of c-di-AMP can be explained by its conversion into cAMP. Therefore, BALB/c mice were immunized with the model antigen β -Gal (15 $\mu\text{g}/\text{dose}$) alone or co-administered with either c-di-AMP or cAMP by the i.n. route. As shown in Figure 32A, animals receiving cAMP as adjuvant showed only a marginal increase in β -Gal-specific IgG-titres, as compared to animals vaccinated with the antigen co-administered with c-di-AMP. Lymphoproliferative assays were then carried out to evaluate the capacity of cAMP to promote cellular immune responses. To this end, splenocytes isolated 42 days after the first immunization were re-stimulated *in vitro* with the β -Gal protein. No proliferative responses were observed in cells from mice receiving β -Gal with cAMP (Figure 32B). This data suggest that under these experimental conditions cAMP does not exert any strong adjuvant effect when administered by the i.n. route. Conversely, it seems unlikely that c-di-AMP derived c-AMP could be responsible for the observed immune modulatory activities of c-di-AMP.

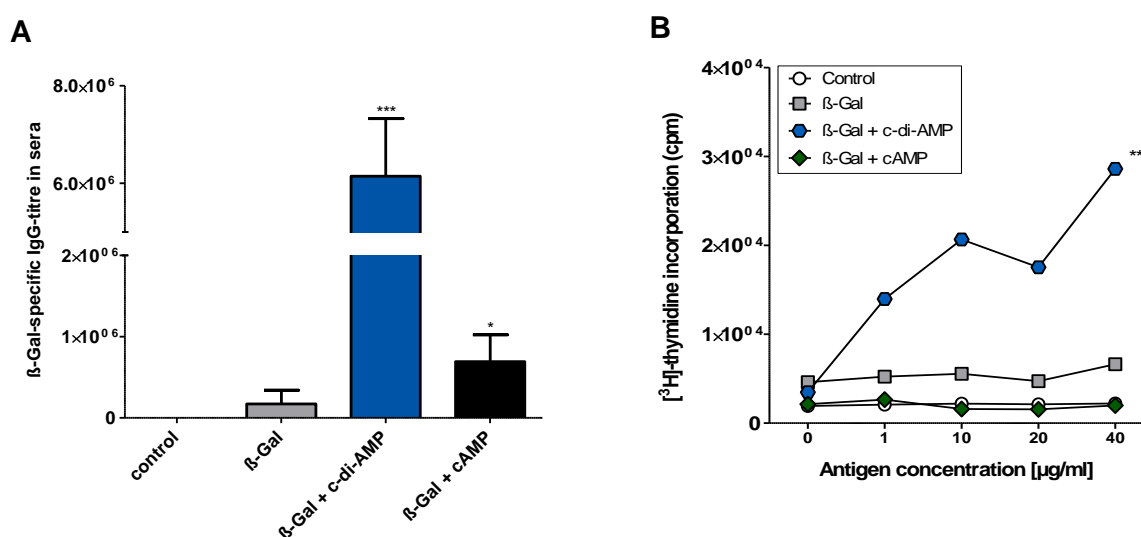


Figure 32: Comparative evaluation of the adjuvant properties of cAMP and c-di-AMP in BALB/c mice vaccinated i.n. route.

A) Systemic humoral immune responses stimulated in BALB/c mice vaccinated using c-di-AMP (5 $\mu\text{g}/\text{dose}$) or cAMP (2.5 $\mu\text{g}/\text{dose}$) as adjuvant. Kinetic analysis of anti- β -Gal IgG responses in sera from BALB/c mice ($n=5$) immunized on day 0, 14 and 28 with PBS (control), β -Gal alone (15 $\mu\text{g}/\text{dose}$) or β -Gal co-administered with 5 $\mu\text{g}/\text{dose}$ of either c-di-AMP or cAMP by the i.n. route. Differences were statistically significant at $p<0.001$ (***) or $p<0.05$ (*) with respect to values in BALB/c mice receiving antigen alone. One representative out of four independent experiments is shown. The results are expressed as end point titres. The S.E.M. is indicated by vertical lines. **(B)** Evaluation of the cellular immune responses stimulated in vaccinated mice. Spleen cells from vaccinated animals were re-stimulated with different concentrations of β -Gal for 96 h. Cellular proliferation was then assessed by determination of the $[^3\text{H}]$ -thymidine incorporated into the DNA of replicating cells. Results are averages of quadruplicates and they are expressed as stimulation index (SI). The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p<0.01$ (**) with respect to BALB/c mice receiving antigen alone. One representative out of three independent experiments is shown.

3.5.2 Intracellular targeting of c-di-AMP

There are different processes by which the cells can uptake substances. The two main forms are by endocytosis or phagocytosis. Besides the molecular signalling induced by c-di-AMP it is also important to understand if and how cells uptake the adjuvant. Therefore, the adjuvant was labelled by different methods, such as Nanogold (NG) for electron microscopy analysis (EM) or with the fluorescent tracer fluorescein for confocal examination.

3.5.4.2. Accumulation of Nanogold-c-di-AMP in vesicles of DCs.

To study the uptake and the detectability c-di-AMP was labelled with Nanogold and incubated with BMDCs for different time points. Nanogold is a small (1.4 nm) and highly uniform gold compound and can be used to label primary amines of c-di-AMP. In order to assess the significance of the resulting observations, the ability of the c-di-AMP-conjugate (NG-c-di-AMP) to stimulate DCs was first investigated by FACS. The c-di-AMP-conjugate was still able to stimulate the DCs in a comparable intensity as the non-conjugated adjuvant (Figure 33).

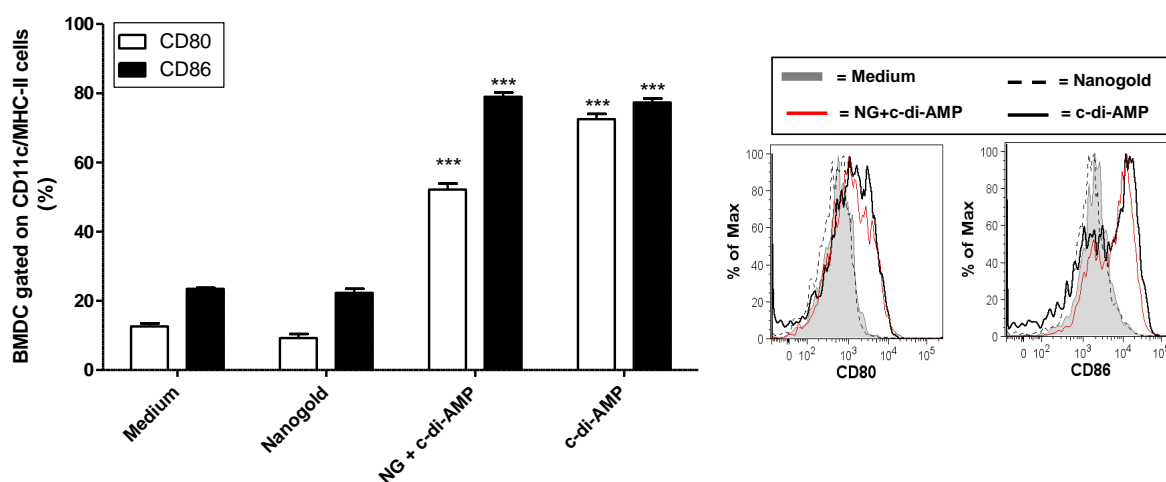


Figure 33: The ability of NG-c-di-AMP to stimulate DCs.

BM-derived murine DCs were incubated with NG-c-di-AMP, c-di-AMP, NG alone and medium as control for 24 h and analysed by flow cytometry. The expression of the co-stimulatory surface markers CD80 and CD86 on CD11c⁺/MHC-II⁺-gated cells is shown. The S.E.M. is indicated by vertical lines. Differences were statistically significant at $p < 0.001$ (***) with respect to untreated BMDCs. One representative out of two independent experiments is shown.

Next, the fate of NG-c-di-AMP in DCs was examined by EM analysis. To this end, DCs were incubated with the conjugate for different time intervals. The Nanogold signal was

enhanced with a silver developer so that the small gold particles were easily visualized by EM.

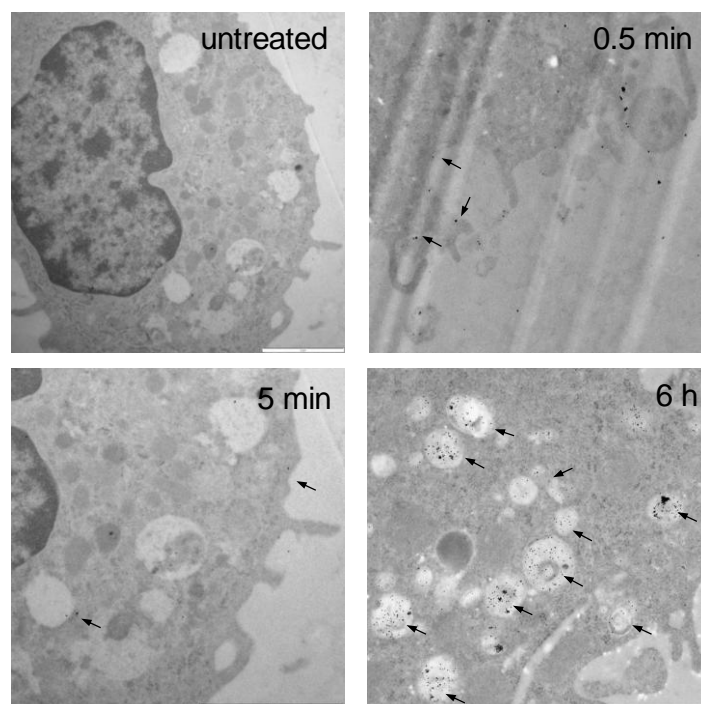


Figure 34: Electron microscopy analysis of Nanogold-c-di-AMP conjugate accumulated in vacuoles of DCs.

EM analyses of BMDC treated with or without NG-c-di-AMP (black arrows) for 0.5 min, 5 min and 6 h after silver enhancement.

The NG-c-di-AMP conjugate was detected already after 0.5 min in the cytoplasm of DCs and accumulated in vesicles with increasing incubation time (Figure 34). After 6 h of incubation with the c-di-AMP-conjugate, DCs showed a high number of intracellular vesicles including the conjugate (Figure 34); however no NG-c-di-AMP could be detected in the nucleus.

3.5.4.3. Examination of the intracellular targeting of 2'-Fluo-AHC-c-di-AMP in BMDCs by confocal microscopy

In order to characterize the intracellular compartments in which resides the c-di-AMP, immune labelling experiments with antibodies directed against the early endosomes marker Rab5 and the late endosomal/lysosomal marker LAMP2 were undertaken. BMDCs were treated with the 2'-Fluo-AHC-c-di-AMP conjugate for 1 h and stained with Hoechst 33258 and Rab5 or LAMP2 and analysed by confocal microscopy. The obtain results confirm the EM

analysis indicating an accumulation of c-di-AMP conjugate in intracellular compartments of DCs after 1 h of treatment. The merged figure shows an accumulation of c-di-AMP in early endosomes labelled with Rab5 (Figure 35).

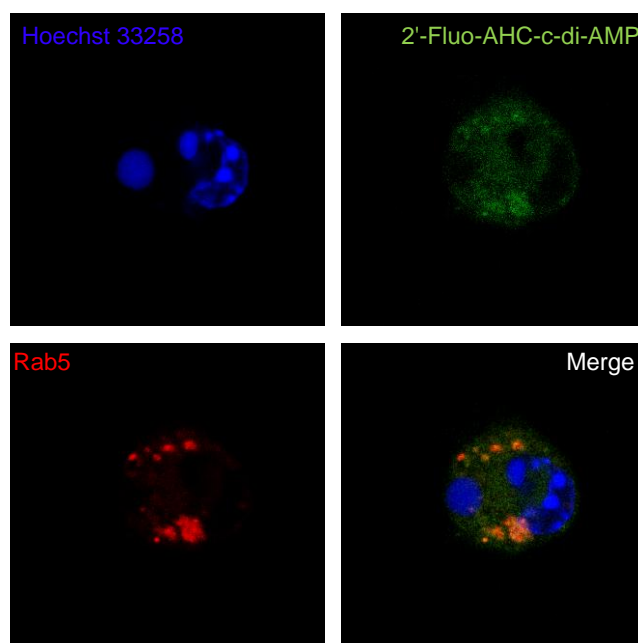


Figure 35: Analysis by confocal microscopy of 2'-Fluo-AHC-c-di-AMP conjugate treated DCs using antibodies specific for Rab5.

BMDCs were treated with 2'-Fluo-AHC-c-di-AMP (green) for 1 h, mounted onto slides, fixed, permeabilized, subjected to indirect anti-Rab5 (Cy3, red) and nuclear (Hoechst 33258, blue) immunostaining and analysed by confocal laser-scanning microscopy. To demonstrate the relative positional distribution of Hoechst-, Cy3- and FITC-labelled moieties, the images collected in the three fluorescence channels were merged. In the overlay mode, subcellular areas in which Cy3 and FITC fluorescence co-localize appear in yellow.

The investigation of late endosomes/lysosomes also indicated an accumulation of c-di-AMP conjugate in the LAMP2 marked vesicles (Figure 36). However, no c-di-AMP-conjugate could be detected in the nucleus.

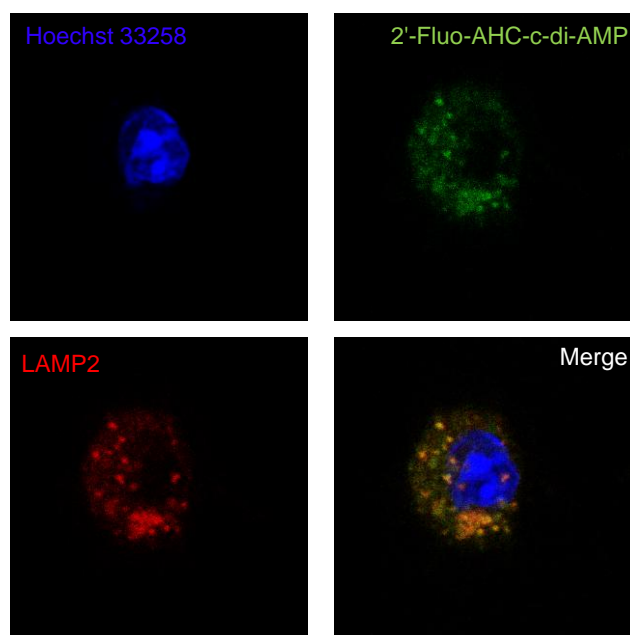


Figure 36: Analysis by confocal microscopy of 2'-Fluo-AHC-c-di-AMP conjugate treated DCs using antibodies specific for LAMP2.

BMDCs were treated with FITC-c-di-AMP (green) for 1 h, mounted onto slides, fixed, permeabilized, subjected to indirect anti-LAMP2 (APC, red) and nuclear (Hoechst 33258, blue) staining and analysed by confocal laser-scanning microscopy. To demonstrate the relative positional distribution of Hoechst-, APC- and FITC-labelled moieties, the images collected in the three fluorescence channels were merged. In the overlay mode, subcellular areas in which APC and FITC fluorescence co-localize appear in yellow.

3.5.3 Induction of type I IFN response by c-di-AMP after *i.n.* administration

Several studies demonstrated that DC activation (e.g. after detection of nucleic acids through TLR or cytosolic sensors) can lead to type I IFN secretion. Type I IFN not only directly inhibits viral infection, but also activates the antiviral functions of NK cells, DCs, B cells and T cells, thereby playing a central role in the initiation and regulation of innate and adaptive antiviral immunity [132, 133]. Thus, the *in vivo* effect of c-di-AMP on type I IFN induction was studied.

To determine the contribution of myeloid or lymphoid cell populations to the IFN- β production after c-di-AMP treatment, tissue/cell subset specific reporter mice were used since these mice allow tissue/cell subset specific replacement of the IFN- β gene by the luciferase reporter in the IFN- $\beta^{+/flox-b-luc}$ mice [134]. These mice were crossed with mice expressing Cre recombinase under various tissue/cell subset specific promoters. CD19cre mice were used to implement the reporter activity in B cells, CD4cre mice in T cells, LysMcre mice in monocytes/macrophages and granulocytes and CD11cre mice in DCs. All reporter mice were heterozygous for both markers to allow normal cellular development as well as potential production of IFN- β in all cells. Comparison of such tissue specific reporter mice

after c-di-AMP treatment with the “global” IFN- β reporter mouse (IFN- $\beta^{+/Δ\beta-luc}$) by whole body *in vivo* imaging demonstrated that T and B cells did not have any apparent impact on the IFN- β production after i.n. administration of c-di-AMP (Figure 37). However, cells in which the LysM- and CD11c-promoter is active, significantly contribute to the IFN- β signal and leads to a maximal IFN response 6 h after administration (Figure 37). Additionally, IFNAR-/- mice were used to investigate the influence of c-di-AMP on type I IFN expression. The obtained results revealed that c-di-AMP is triggering a strong local time-dependent induction of the IFN- β genes in IFNAR-/- mice comparable to the signal of the global IFN- β reporter mice with an average radiance of 10^5 p/sec/cm²/sr (Figure 37B).

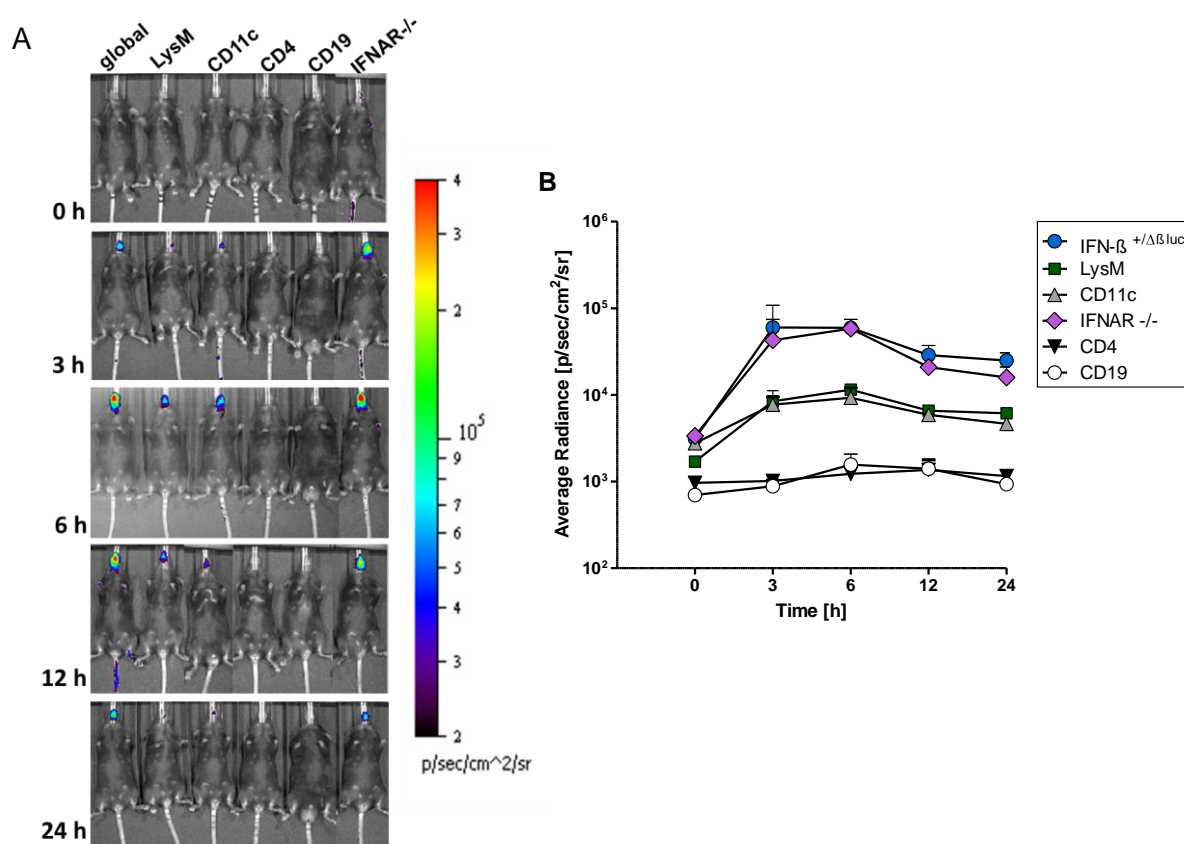


Figure 37: Induction of IFN- β genes after administration of c-di-AMP.

Mice of indicated phenotypes in the C57BL/6 genetic background were i.n. treated with c-di-AMP (5 μ g/dose): “global” stands for IFN- $\beta^{+/Δ\beta-luc}$; “CD4”, “CD19”, “LysM” and IFNAR-/- stand for IFN- $\beta^{+/flox-b-luc}$ x CD4cre, IFN- $\beta^{+/flox-b-luc}$ x CD19cre, IFN- $\beta^{+/flox-b-luc}$ x LysMcre and IFNAR knock out, respectively. **A)** At the depicted time points after i.n. administration of c-di-AMP (5 μ g/dose), mice were injected with luciferin (i.v.) and luciferase activity was visualized in the IVIS 200 imaging system. Low signals are due to quenching of the bioluminescent light by melanin in fur and skin of the C57BL/6 mice. **B)** Quantification of *in vivo* imaging by measuring of luminescence intensity of the mice with indicated phenotypes shown in A) at the depicted time points. The experiment was done twice with 2-3 animals per group. Results are expressed as average of radiance. S.E.M. is indicated by vertical lines.

3.5.4 Phosphorylation of MAPK is activated in c-di-AMP treated MΦs and DCs

To determine whether c-di-AMP could activate intracellular signalling, the effect of c-di-AMP on the activation of MAPK in MΦs (the macrophage like cell line J774.A1) and primary BMDCs was investigated. To this end, MΦs or BMDCs were treated with 5 µg/ml of c-di-AMP for different time intervals (5, 20 or 30 min), the cells were then lysed and a Western blot based on the Proteome Profiler Human Phospho-MAPK Array was performed.

The results showed a phosphorylation of extracellular signal-regulated kinase 1/2 (ERK 1/2), and p38α MAPK (MAPK14) even 5 min after c-di-AMP stimulation of MΦs. These levels of phosphorylation gradually decreased 20 and 30 min after treatment. Further MAPKs such as Jun N-terminal kinase1 (JNK1) and JNK2 were slightly increased after 5 and 20 min. However, phosphorylation of JNK3, p38β, γ and δ MAPK was not detected in MΦs at all (Figure 39).

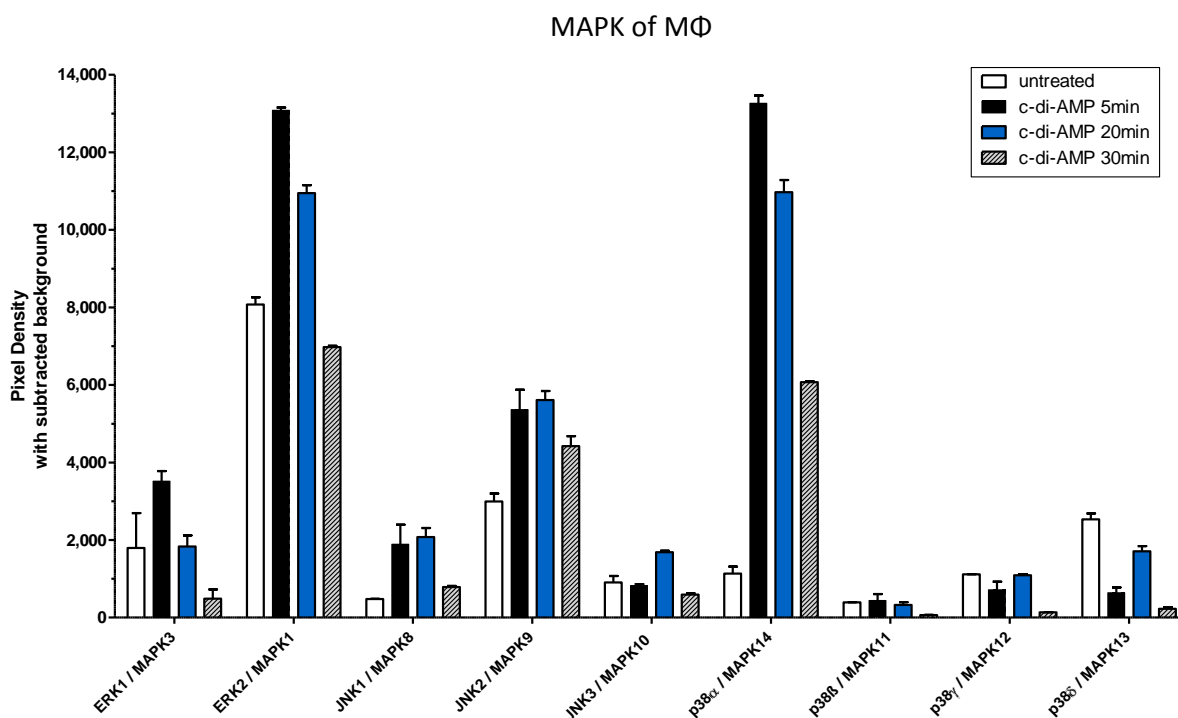


Figure 38: c-di-AMP induces MAPK signalling in macrophages.

Activation of MAPK pathways in a macrophage cell line (J774A.1). MΦs were incubated in the absence or presence of c-di-AMP (5 µg/ml) for 5 min, 20 min or 30 min. Following exposure to c-di-AMP, MΦs were harvested to make cell lysates. Identical amount of cell lysates was separated on a membrane and analysed by Western blot based Proteome Profiler Human Phospho-MAPK Array Kit.

The analysis of phosphorylated MAPK in cDCs showed also a slight activation of the p38α MAPK 5 min after stimulation with c-di-AMP, with the level of phosphorylation increasing strongly after 20 min and decreasing slightly at 30 min. However, the

phosphorylation of ERK1/2 is shown to be only slightly activated 30 min after c-di-AMP treatment. A marginal phosphorylation is also seen in JNK1 and 3, p38 β , γ and δ MAPK after 30 min of stimulation (Figure 39). Other proteins, such as JNK2, showed suppression after the stimulation with c-di-AMP. In general, the MAPK phosphorylation in M Φ s is much stronger than in DCs after stimulation with c-di-AMP, and distinct patterns were observed in each cell type. The rapid modification of the phosphorylation pattern suggests that a direct rather than an indirect mechanism relaying on gene activation is responsible for the observed changes.

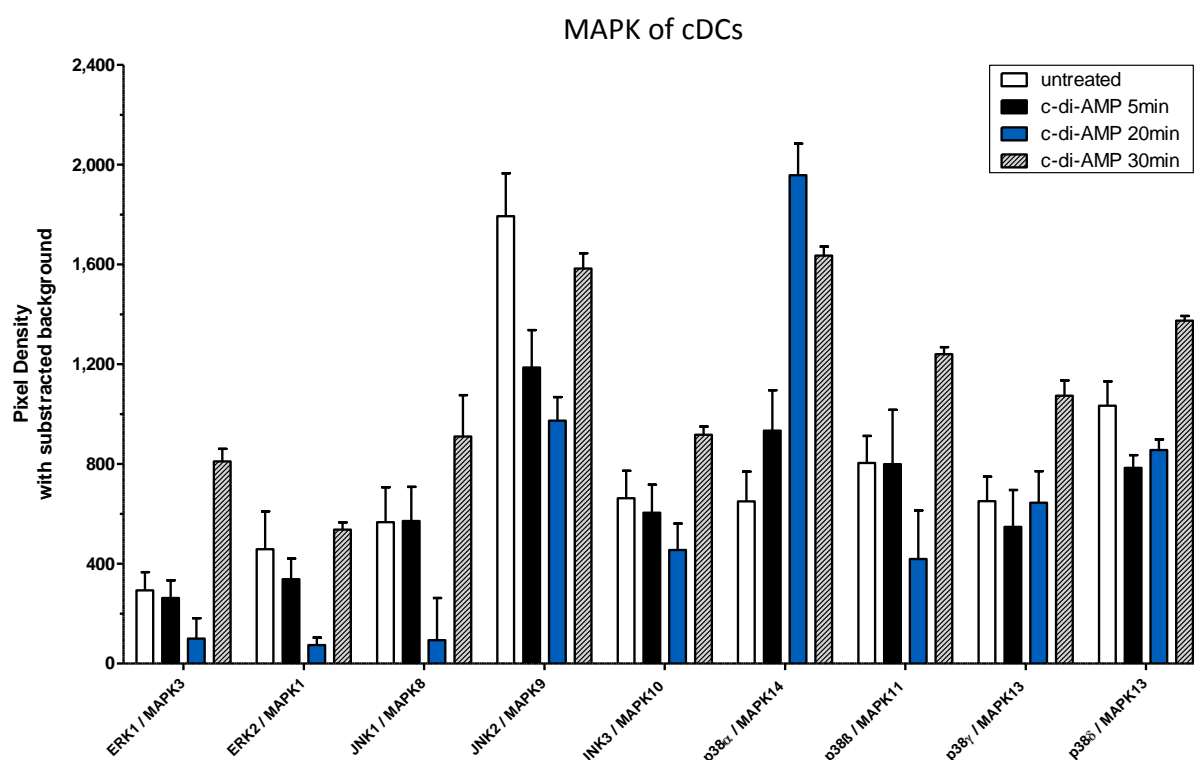


Figure 39: c-di-AMP induces MAPK signalling in cDCs.

Activation of MAPK pathways in a BMDCs. DCs were incubated in the absence or presence of c-di-AMP (5 μ g/ml) for 5 min, 20 min or 30 min. Following exposure to c-di-AMP DCs were harvested to make cell lysates. Identical amount of cell lysates was separated on a membrane and analysed by Western blot Western blot based Proteome Profiler Human Phospho-MAPK Array Kit.

4. CHAPTER: DISCUSSION

4.1. Cyclic di-nucleotides exhibit high potential as adjuvants for the development of mucosal vaccines.

4.1.1. Requirement of new adjuvants

In recent years we have assisted to the advent of new approaches and technologies, which have in turn facilitated the identification of candidate antigens for developing novel subunit vaccines. For example, the promising genomic-based approach known as “reverse vaccinology” has been exploited to identify antigens, which have been translated into the clinical development pipeline [135-139]. Thus, in the post-genomic era the identification of suitable candidate antigens does not represent a major bottleneck any longer. However, subunit vaccines are poorly immunogenic, raising the need for adjuvants able to promote the elicitation of responses of appropriate quality and strength. Therefore, it is becoming obvious in the field of vaccinology that for vaccine development, the availability of an optimal adjuvant is as important as the selection of the antigen. This need is becoming more pressing when issues such as antigen sparing in the context of pandemic threats, immune modulation for therapeutic vaccines, or vaccines tailored for specific population groups (e.g. new-borns, elderly) are considered.

Most pathogens enter the host via the mucosal tissues; however, most licensed vaccines are administered via parenteral route [51]. This approach mainly leads to the elicitation of systemic responses. Thus, it is not possible to block the agents at the portal of entry, thereby reducing their capacity to colonize the host. Hence, the induction of immune responses at both systemic and mucosal levels represents a major asset for a vaccine against a pathogen which is restricted to, or need to transit across the mucosa in order to cause disease. In fact, the efficient stimulation of immune responses at the portal of entry would not only prevent disease, but also infection (i.e., colonization). In addition, vaccination by the mucosal route is more accepted by the public and it is associated with a lower risk for cross contamination or appearance of side effects [140]. This can be achieved by administering the antigens by a mucosal route. However, purified antigens are even less immunogenic when administered by this route, due among other reasons, to their clearance, degradation and/or poor penetration [141]. This bottleneck can be overcome by the use of mucosal adjuvants. However, there are even less candidate adjuvants exhibiting this feature.

The mucosal immune system is compartmentalized and there is no physical connection between different mucosal territories [52]. Nevertheless, our current knowledge

on the structure and function of the mucosal immune system, the interplay between its cellular components, the role of locally produced soluble mediators, the linkage between different mucosal niches in a fully integrated network and their bridging with the systemic compartment are, at the very best, incomplete. Therefore, it is critical to elucidate the underlying mechanisms to the elicitation of immune responses following vaccination by the mucosal route. This would allow exploiting the full potential of this strategy for the establishment of prophylactic and therapeutic interventions against infectious and non-infectious diseases.

Promising mucosal candidate adjuvants are the second messengers c-di-GMP, c-di-AMP and c-di-IMP (Figure 3). The c-di-GMP was first identified in *Gluconacetobacter xylinus* as a secondary metabolite involved in the regulation of cellulose production [98]. Additional studies by Karaolis *et al.* and other groups showed that exogenous c-di-GMP reduces *in vitro* cell-cell interactions and biofilm formation of *S. aureus* and also demonstrated that treatment with c-di-GMP attenuates *S. aureus* infection *in vivo* in a mouse model [102, 103, 108, 142-144]. Unexpected, they found that c-di-GMP has no apparent inhibitory or bactericidal effect on *S. aureus in vitro*, thereby suggesting that the observed reduction in colonization by biofilm-forming *S. aureus* strains was due to a biological effect on the host immune system [110, 145]. In fact, in this doctoral thesis it is shown that *in vitro*, c-di-GMP and to a greater extent c-di-AMP stimulates the production of nitric oxide by pre-activated murine macrophages at a concentration of 200 and 1.6 ng/ml, respectively (Figure 6).

Subsequent reports described another compound of the cyclic di-nucleotide family, c-di-AMP (Figure 3), which serves as a second messenger signalling for DNA integrity in *B. subtilis* during sporulation [129]. Additional studies showed that c-di-AMP is widespread in bacteria and archaea, suggesting that this compound may act as a second messenger in response to various cues besides branched DNA [112]. More recently, Woodward *et al.* identified c-di-AMP as a secreted molecule by *L. monocytogenes*, which is able to trigger the cytosolic host response of innate immunity [113]. Since they seem to be crucial for several key bacterial processes, it can be assumed that these molecules could serve as danger signals recognized by the host immune system. It can be also argue that other structurally related, although chemically distinct compounds could also act as danger signals able to evoke an immune response in the host. An example of this is the c-di-IMP (Figure 3), which was synthesized out of the parental compound c-di-AMP through adenosine-deaminase.

An in depth side-by-side characterization of the immune modulatory properties of the three second messengers c-di-GMP, c-di-AMP and c-di-IMP after mucosal administration was therefore performed. In the focus of this doctoral thesis was first the identification of the effector functions of the three cyclic di-nucleotides and their potency as mucosal adjuvants to select the most promising compound for further development (i.e. c-di-AMP). Second, the

analysis of the targeted cells and effector functions. Third, the identification of the underlying mechanisms of action and/or putative molecules involved in the signalling transduction events leading to the exertion of its adjuvant properties. To this end, the *in vitro* and *in vivo* influence of c-di-AMP on the activation and maturation of different immune cells was investigated (e.g., DCs, MΦs, B cells, T cells).

4.1.2. *Stimulation of antigen-specific immune responses by the c-di-nucleotides*

In vivo studies were carried out to evaluate their adjuvant capacities using β-Gal or OVA as model antigens. The presence of c-di-GMP, c-di-AMP or c-di-IMP in the formulation resulted in a significantly improved stimulation of antigen-specific immune responses at mucosal level. More specifically, high titres of β-Gal-specific IgG were detected in sera of mice vaccinated with c-di-AMP (>1:6,000,000), c-di-GMP (1:4,000,000) or c-di-IMP (1:3,500,000) as adjuvants in comparison to sera from control mice receiving β-Gal alone (<1:2,000), as shown in Figure 7. Analysing sera and lavage samples of C57BL/6 mice vaccinated with OVA alone or co-administered with c-di-AMP, c-di-GMP or c-di-IMP elevated a comparable antigen-specific IgG and IgA expression (Figure 11). Furthermore, the co-administration of c-di-nucleotides resulted in enhanced antigen-specific mucosal immune responses, not only locally in the nose or lung, but also at distant mucosal territories, such as in vaginal secretions (Figure 8). Similar results were obtained when a SC-specific ELISA was implemented to rule out that the obtained results were in part derived from Ig transferred into the lumen by transudation. This suggests that efficient activation, migration and homing of precursors are taking place.

Cellular proliferative responses were also enhanced in mice vaccinated using c-di-AMP (SI >20), c-di-IMP (SI >14), or c-di-GMP (SI > 7) with respect to those receiving the β-Gal antigen alone (SI <3; Figure 9). The analysis of sera from animals vaccinated with the cyclic di-nucleotides revealed the presence of high β-Gal specific IgG1 titres, thereby indicating the stimulation of Th2 cells. However, significant levels of antigen-specific IgG2a were detected in sera, suggesting that Th1 cells are also activated (Figure 12). Moreover, the evaluation of the cytokines secreted by lymphoid cells from vaccinated mice showed that vaccination using c-di-nucleotides as adjuvants resulted in an enhanced secretion of IL-2, IL-4, IL-17 and IFN-γ (Figure 13). Re-stimulated splenocytes derived from mice receiving c-di-nucleotides also showed an enhanced expression of soluble factors such as IFN-γ, TNF-α, IL-2, IL-1, IL-4 IL-5 IL-6 and IL-10 which act as attractants of naïve and effector T cells, as well as inducers of T cell differentiation (e.g. IL-17). IL-17 is a key molecule in the recruitment of neutrophils and DC maturation [54-60]. This explains at least in part the observed immune potentiation with

enhanced maturation of various cell subsets, promotion of T cell polarization and development of B cells.

Taken these results together, c-di-GMP, c-di-AMP and c-di-IMP induced a balanced Th1/Th2/Th17 response pattern. This profile is particularly attractive, since most adjuvants described up to now promote either Th1 or Th2 biased responses. The stimulation of a more balanced Th1/Th2/Th17 pattern after immunization with cyclic di-nucleotides as adjuvants, suggests that they are more flexible molecules, which would allow fine-tuning immune responses in the context of a broader range of clinical applications.

Although cyclic di-nucleotides have similarities in some general features of their molecular structure, there are clear chemical and biological differences that make each of them a unique compound. Beyond their being distinct chemical entities, the *in vivo* studies show the stimulation of a balanced Th1/Th2/Th17 immune responses after the administration of the c-di-nucleotides by intranasal route. However, there are also some differences in the potency of these small molecules, suggesting that c-di-AMP exerts the most potent adjuvant activity. This is further supported by the distinct functional biological properties of cyclic di-nucleotides observed on pre-activated macrophages *in vitro* (Figure 6). Moreover, microarray studies performed with MΦs stimulated with cyclic di-nucleotides revealed a distinct transcriptional profile suggesting a different mode of actions (data not shown). The balance between differences in structure and effector functions needs further elucidation in order to exploit these compounds for fine-tuning responses to vaccine antigens.

4.1.3. *The novel adjuvant c-di-AMP is acting preferentially plainly on DCs*

Stimulation of innate immunity is initiated by the interaction of PAMPs with PRR, present on immune cells [146]. Of special importance are the professional APCs - MΦs, DCs and B cells – which initiating the cascade of events that result in the development of immunity [147, 148]. Professional APCs express a wide variety of PRR. These receptors are used as sensors for pathogens and also sample antigens in their microenvironment. Binding of PAMPs by PRR induces a DC activation that comes along with the up-regulation of MHC antigens and co-stimulatory molecules, such as CD40, CD80 and CD86 [149]. The up-regulation of these molecules is accompanied by the differentiation of immature into mature DCs, MΦs or B cells [150].

When investigating the effect of c-di-AMP on mouse DCs, MΦs and splenic B cells *in vitro*, a clear up-regulation of activation markers has been observed in DCs and MΦs, but not in B cell (Figure 16 and 17). Thus, exogenous administered c-di-AMP seems to have a strong impact on the maturation and activation of DCs and MΦs, thereby leading to a downstream signalling which was expected to result in improved innate and adaptive

immunity. More important, this is not restricted to the murine system, since it could be demonstrated that human immature DCs and MΦs stimulated with c-di-AMP strongly increase the expression of maturation and activation markers (MHC-II, CD80/CD86, CD83; Figure 26). This makes c-di-AMP a true candidate adjuvant for clinical use in humans.

Following maturation, DCs play an important role in the activation of both the innate and adaptive immune system. After sampling antigens in the periphery and activation, mature DCs migrate to secondary lymphoid tissues, where they interact with different immune cells, thereby leading to the activation of lymphocytes and the stimulation of antigen-specific adaptive responses. In fact, co-stimulatory and adhesion molecules represent critical co-receptors for T cell priming in the context of both MHC class I and II molecules [151]. DC activated T cells can complete the immune response by interacting with other immune cells, such as B cells for antibody formation, macrophages for cytokine release, and targets for lysis [150]. The investigation of the capacity of murine and human c-di-AMP-stimulated DCs to activate T cells resulted in an enhanced proliferation of both CD8⁺ and CD4⁺ T cells (Figure 19 and Figure 27). However, as expected and desired, no direct induction of murine T cells could be observed after c-di-AMP treatment (Figure 20). The excellent performance of c-di-AMP in *in vitro* experiments led us to performing studies to assess the effector functions of c-di-AMP on DCs and MΦs *in vivo*. The results obtained in mice treated by i.n. route with c-di-AMP for 24 h showed an activation and maturation of DCs and, to a lesser extent, MΦs (Figure 28 and Figure 29).

The two major DC subsets in mice are CD11b⁺, CD11c⁺ cDC and CD11b⁻, B220⁺ pDC. The examination of these DC subsets *in vitro* showed a stimulation of all subsets, cDCs (mDCs and IDCs) and pDCs, after the treatment with c-di-AMP. However, cDCs showed a much higher up-regulation of the co-stimulatory molecules of the B7 family (CD80 and CD86) than pDCs (10 versus 1.5 fold, respectively). Nevertheless, the *in vivo* results showed an up-regulation of CD86 and MHC-II in both cDCs and pDCs from NALT and cLN after i.n. administration of c-di-AMP (Figure 30 and Figure 31).

Mature DCs release large amounts of cytokines, such as IL-12 or IL-10, which shape the local microenvironment and can promote the development of Th1 and Th2 immune responses, respectively [147, 152]. The ability of c-di-AMP to stimulate DCs resulted in a high intracellular production of IL-12p40. It seems that cDCs are the main IL-12 producers after c-di-AMP stimulation, since they showed a 60-fold higher IL-12 production than pDCs. The IL-12 production appears to be c-di-AMP dose-dependent, being apparently CD8α⁺ DCs (IDCs) the main producers (Figure 24). However, IL-12 production can be affected by exogenous factors, such as microbial infections or stress, suggesting that IL-12 production strongly depends upon the general status of the environment in which DCs are located [153]. Furthermore, it is theoretically possible, because IL-12p40 is produced in large excess

compared with IL-12p35, that it is used to form IL-23 [39]. Follow up studies have subsequently shown that IL-23 is in fact, functionally distinct from IL-12, as it not only acts on Th1 cells, but also stimulates Th17 cells to produce IL-17 [39]. This might in part explain the observed induction of Th17 cells.

On the other hand, IL-10 is a potent anti-inflammatory cytokine that can act as a feedback regulator of the inflammatory response to many microbial stimuli [154]. In addition, IL-10 can inhibit or modulate the release of IL-12 and the effect of IL-12 on T cells, thereby down-regulating Th1 responses. The balance between IL-10 and IL-12 is therefore important in the induction of T cell immunity [155, 156]. Interestingly, the intracellular IL-10 production on cDCs showed a 2-fold increment after c-di-AMP treatment in respect to untreated DCs (Figure 25). These investigations are consistent with the obtained balanced Th1/Th2 immune response.

4.1.4. Putative mechanism of action of c-di-AMP

During the last years, it became clear that adjuvants cannot only potentiate immune responses, but can also modulate them. Thus, the understanding their mechanism of action is critical to make optimal use of them, as well as to foresee potential safety issues. Therefore, it is important to identify cellular receptors and elucidate downstream signalling cascades. Although there is an increasing body of experimental evidence dissecting the signalling events triggered by c-di-nucleotides in prokaryotes [105-107, 112, 157, 158], much less is known about the effector functions on immune cells. Initial experiments performed by Karaolis *et al.* using TLR-expressing cell lines suggested that immune activation by c-di-GMP does not involve TLRs 1–9 or NODs 1 and 2 [111]. This was further confirmed by both *in vitro* studies showing the independency of TLR and RLR signalling by using MyD88/TRIF (Myeloid differentiation primary response gene 88/toll-interleukin-1 receptor [TIR]-domain-containing adapter-inducing interferon- β) and MAVS (mitochondrial antiviral signalling) deficient macrophages [159], and *in vivo* immunization studies using MyD88 and TIRAP (TIR-domain containing adaptor protein) knockout mice (unpublished data). On the other hand, the potential contribution of the NOD-like receptor pathway was ruled out by the intact responsiveness of macrophages from mice deficient for *Rip2* $-/-$, *Nalp3* $-/-$ and *Nod1/2* $-/-$ to c-di-GMP [159]. Additional work suggested that c-di-GMP can be acting, at least in part, by a novel mechanism leading to transcriptional activation of type I IFN via CSP [159]. Interestingly, studies on *L. monocytogenes* secreted c-di-AMP also showed TLR and RLR independency and postulated a comparable induction of host CSP [113]. The CSP was initially described as an innate host surveillance mechanism, which specifically distinguishes

bacteria in the cytosol from bacteria in the vacuole, leading to the up-regulation of IFN- β [160-162].

Several studies demonstrated that DCs can detect nucleic acids through TLR or cytosolic sensors, thereby resulting in the expression of type I IFN [133, 163]. Type I IFNs derived from pDCs do not only directly inhibit viral infection, but also activate the antiviral functions of other DC subsets, NK cells, B cells and T cells, thereby initiating and orchestrating innate and adaptive antiviral immunity [132, 133]. Furthermore, type I IFNs increase the ability of DCs to cross-present exogenous antigens to CD8⁺ T cells and promote their clonal expansion [164, 165], as well as their capacity to induce the differentiation of naive T cells into Th cells [166]. Therefore, c-di-AMP was investigated for its ability to induce type I IFNs following i.n. administration. The obtained data revealed a very fast induction of a reporter for type I IFN- β genes 6 h after treatment. Furthermore, by a genetic approach the cells that are responsible for IFN- β production during intranasal c-di-AMP treatment were investigated. It was shown that IFN- β is almost exclusively produced by CD11c⁺ and LysM expressing cells in the nasal cavity (Figure 37). Other mice, such as CD19cre mice used to implement the reporter activity in B cells, and CD4cre mice in T cells did not show any IFN- β production after c-di-AMP treatment. Furthermore, these studies show that in the absence of the IFNAR component, there is still an IFN- β signalling after the treatment with c-di-AMP, which is as strong as the signalling in global reporter mice. This positive feedback can be due to the fact that c-di-AMP is utilizing some factors or pathways stimulating the type I IFN response such as IRF-3, as reported by Woodworth *et al.* [113]. Further *in vivo* analyses would be necessary for the understanding of the functions of c-di-AMP in the context of type I IFN signalling. Hence, treatment with c-di-AMP not only activates DCs, but also triggers type I IFNs. This would in turn strengthen its potential as adjuvant at both systemic and mucosal level. However, the use of the LysMcre mouse does not allow distinction between myeloid cell populations, although lower expression in CD11c⁺ cells was reported [167]. Nevertheless, the global emerging picture from these findings confirms the further results showing that c-di-AMP is primary targeting DCs but also M Φ s. Thus, c-di-AMP can be considered as a danger signal which triggers a cascade of immune signals, thereby leading to improved innate and adaptive immunity.

Interestingly, there are *in vitro* studies showing the induction of similar transcriptional profiles in cells stimulated by cyclic di-nucleotides and DNA. Both are able to trigger type I IFNs and co-regulated genes via induction of Tank-binding kinase 1 (TBK1) and its substrate the IRF-3, as well as nuclear factor NF- κ B and MAP kinases [113, 159, 168]. On the other hand, *in vivo* studies showed that c-di-GMP activates both IRF-3 and IRF-7 [159]. However, cyclic di-nucleotides are not signalling through the cytosolic DNA sensor DAI (DNA-dependent activator of IRFs) [29, 147], as it is the case for DNA (Figure 39). Thus, great

importance should be attached to the MAPK signalling pathways, which play important roles in many cellular processes, including growth, differentiation, apoptosis, and the stimulation of immune responses. It has been reported by McWhirter et al. that c-di-GMP is able to activate all three MAPK pathways in mouse bone marrow macrophages, the ERK, p38 kinase, and JNK [147]. Interestingly, activation of the p38 kinase is also required for the induction of gene expression by the CSP. Studies shown in Figure 34 demonstrated that stimulation with c-di-AMP of the murine macrophage-like cell line J774A.1 also results in the activation of p38 α MAPK, ERK, and JNK kinases. In contrast, analysis of MAPK of cDCs resulted in a much lower phosphorylation level of p38 α MAPK, followed by slightly activated ERK and even a suppression of JNK (Figure 39). More interestingly, the activation of MAPK was induced later in cDCs than in M Φ s. However, these are preliminary results, it would be necessary to confirm them by more in depth analysis, for example with the BD™ Phosflow technology, which makes it possible to reveal intracellular data on protein phosphorylation by flow cytometry. This could in turn close the circle, since protein kinases are involved in type I IFN signalling, and deliver anti-apoptotic and pro-survival signals [158]. Recent studies showed that an ER-localized stimulator of interferon genes (STING) is required for the type I IFN response to c-di-GMP (both *in vitro* and *in vivo*) and c-di-AMP (only tested *in vitro*) [159, 160]. Former studies showed that the STING protein is critical for regulating the production of IFN in response to cytoplasmic DNA virus [161] (Figure 40).

Although there are several distinct subtypes of DCs, they all share key features. Namely, they continuously sample their surroundings ingesting antigens by endocytosis (using phagocytosis, receptor-mediated endocytosis, and pinocytosis) [22-24]. As it was reported in the studies on *L. monocytogenes* secreted c-di-AMP in the cytosol, we performed detection studies of the adjuvant in DCs [113]. The electron microscopic analysis showed a time-dependant increment of c-di-AMP aggregation in the cytosol, mostly in vesicles. Importantly, no c-di-AMP could be detected on the surface during the first 5 min (Figure 34), suggesting that the adjuvant is not binding to any surface receptor, which means that the molecule is probably passing the membrane or is taken up by pinocytosis. Moreover, no c-di-AMP could be detected in the nucleus. The confocal analyses confirm the EM data, showing that c-di-AMP aggregates in vacuoles containing endosomal and lysosomal markers (Figure 35 and Figure 36). Endocytosis signalling was originally viewed as a mechanism that delivered receptors to degradation or recycling pathways. It is now clear that diverse families of receptors can signal from the endosomal network to control essential cellular processes [169]. These endosomal signals differ from those originating from receptors located on the plasma membrane, both mechanistically and temporally, and endosomal signalling is tightly regulated by mechanisms that are not fully understood. Moreover, late endosomes have been proposed to serve as a site of assembly of signalling complexes that do not contain

receptors [170]. For example, the MAP kinase scaffold protein MP1, capable of binding to MEK1 and ERK1/2, is associated with the late endosomal protein p14 [91]. Knockdown of p14 in human cells and knockout of p14 in mouse demonstrated the importance of this protein for full activation of ERK1/2 [91, 171]. However, further experiments are needed to examine the signalling molecules in the vesicles of c-di-AMP-treated DC, as well as for identifying the binding partners of c-di-AMP.

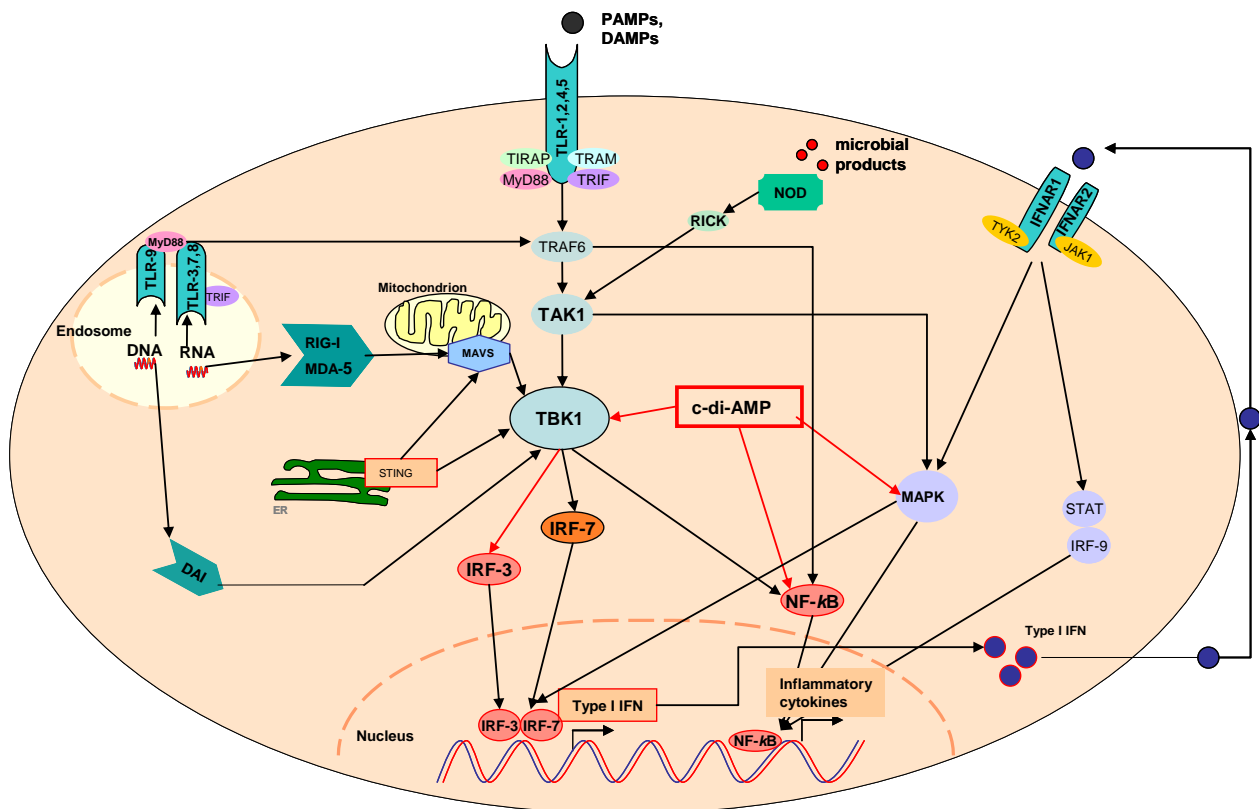


Figure 40: Putative intracellular cascades activated by cyclic di-nucleotides.

In this schematic representation, TLRs are separated in two major groups, those associated to the membrane and those located in the endosomal compartment. For the sake of clarity, in this scheme there is no discrimination between the different TLR at either the membrane or endosomal compartment. The membrane-bound TLRs (TLR-1, TLR-2/1, TLR-2/6, TLR-4 and TLR-5) detect PAMPs and DAMPs on the cell surface and bind to specific TIR domain containing adaptors, such as TRIF, MyD88, TIRAP and TRAM. Other TLRs, such as TLR-3, TLR-7 and TLR-8, are localized in intracellular vesicles and recognize RNA, whereas the intracellular TLR-9 recognizes DNA. The important players downstream in these signalling cascades are TRAF6, TAK1 and TBK1, which in turn phosphorylates IRF-3 and IRF-7, leading to their homo-dimerization and translocation into the nucleus, where they drive transcription of IFNs. This signalling cascades result in the activation of NF-κB and MAPK, which in turn are leading to the production of pro-inflammatory cytokines and type I IFN. On the other hand, TLR-independent recognition of PAMPs is mediated by the intracellular receptors NLRs (NODs) and RLRs (RIG-I, MDA-5) present in the cytosol, which after activation trigger a subset of responses, which are similar to those promoted by TLRs. Exogenous or viral dsRNA is

recognized by the RNA helicase RIG-I (or MDA-5), and signals through the mitochondrial antiviral signalling adaptor MAVS (also known as IPS-1), which activates TBK1, thereby leading to phosphorylation of IRF-3, NF- κ B release, translocation of the transcriptional regulators and gene induction. DNA is sensed by DAI, leading to activation of the same TBK1/IRF pathway as RIG-I/MDA-5. The ER-localized STING protein was shown to be critical for regulating the production of IFN in response to cytoplasmic DNA virus. *In vitro* and *in vivo* studies suggest that c-di-AMP are sensed through a cytosolic pathway leading to type I IFN induction. The induction of type I IFNs by c-di-nucleotides is dependent on TBK1/IRF-3 signalling, although it is independent of known cytosolic receptors. The adaptor molecule STING also seems to be required for the type I IFN responses induced by c-di-nucleotides. Red lines: putative c-di-nucleotide driven pathways for which strong experimental evidence exists. Black lines: non c-di-nucleotides driven pathways.

CONCLUSIONS

The emergence of many infectious diseases together with the unavailability of adequate therapeutic options emphasizes the need for developing new or more efficient vaccines. Modern subunit vaccines are poorly immunogenic and demand the incorporation of adjuvants in the formulation to improve the immunogenicity of purified antigens. In this thesis, it was demonstrated that the cyclic di-nucleotides c-di-GMP, c-di-AMP and c-di-IMP are promising candidates as mucosal adjuvant. It was shown that i.n. immunization with cyclic di-nucleotides co-administered with model antigens promotes the stimulation of a mixed Th1/Th2/Th17 immune response. The experiments presented here have also shown that c-di-AMP is giving a stronger immune response in comparison to c-di-GMP or c-di-IMP. *In vitro* studies showed a strong impact of c-di-AMP on both murine and human DCs. More in depth analysis revealed a strong up-regulation of activation markers of murine cDCs, both *in vitro* and *in vivo*, indicating them as a primary cell target. Functional studies on the mechanism of action showed that c-di-AMP is located in a vesicular compartment of DCs and that it is able to trigger the induction of IFN- β and IL-12 production by DCs and M Φ s. The observed activation of MAPK pathways (e.g. p38 α , MAPK, ERK), suggests these pathways can be regulated by intravesicular signaling of the adjuvant. The possibility to generate synthetic cyclic di-nucleotides as well-defined chemical entities opens new ways to stimulate a broad spectrum of effector cells and clearance mechanisms following vaccination by the mucosal route. Up to now, preclinical studies suggest that cyclic di-nucleotides would also exhibit an adequate safety profile; however, their true potential in terms of safety and efficacy for human vaccine development remains to be proven in the field.

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Publikationen

Libanova,R.; Becker, P.D.; Guzman,CA.; (2011) "Cyclic di-nucleotides: new era for small molecules as adjuvants" Microbial Biotechnology

Libanova,R.; Ebensen,T; Schulze,K; Yevsa,T; Morr,M; Guzman CA. (2011). "Bis-(3',5')-cyclic dimeric adenosine monophosphate: Strong Th1/Th2/Th17 promoting mucosal adjuvant." Vaccine 29(32): 5210-20.

Libanova,R.; Ebensen,T., Schulze,K.; Bruhn,D.; Norder,M.; Yevsa,T.; Morr,M.; Guzmán,CA.* (2010). "The member of the cyclic di-nucleotide family bis-(3', 5')-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant." Vaccine 28(10): 2249-58.

Ebensen,T.; **Libanova,R.;** Guzman,CA.; (2010). Bis-(3',5')-cyclic di-GMP: promising adjuvant for vaccine design.: The Second Messenger Cyclic di-GMP. Wolf, A.J.; Visick,K.L.I.; (Ed.). Washington, D.C., USA: ASM Press, 311-319.

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